DOCTORAL THESIS

Non-Neuronal ATP: Regulation of Release and Action in the Bladder

Grundy, Luke

Award date:
2014

NON-NEURONAL ATP: REGULATION OF RELEASE AND ACTION IN THE BLADDER

PhD Thesis

By

Luke Alexander Grundy

Faculty of Health Science and Medicine

Bond University

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy by Research

April 2014

SUMMARY OF THESIS

This thesis investigates the mechanisms involved in sensory signalling from the mouse urinary bladder. Sensory afferent firing is essential in the initiation of the micturition reflex and ultimately regulates the cycle of bladder filling and bladder emptying. Whilst bladder contraction and efferent function have been studied extensively, the processes that determine afferent signalling remain elusive and two distinct pathways are currently thought to underlie mechanotransduction: direct gating of mechanosensitive afferents in the bladder wall during bladder stretch, and an indirect mechanism via the release of mediators from the urothelium. Understanding more about bladder afferent transduction mechanisms may lead to the development of novel treatments for lower urinary tract disorders in which symptoms are associated with the filling phase of micturition such as overactive bladder and interstitial cystitis.

Mechanisms underlying bladder mechanosensation were investigated both directly, using an in vitro afferent nerve recording technique which allowed the concurrent recording of intravesical pressure and afferent nerve activity, and indirectly, examining urothelial mediator release and intracellular calcium signalling of urothelial cells and isolated DRG neurons. Using a combination of mechanical, pharmacological and genetic tools, a role for P2X, P2Y, TRPV1 and NK2 receptors in influencing mechanosensitivity was investigated.

Experiments employing pharmacological blockade or genetic deletion of the TRPV1 receptor implicated TRPV1 in bladder mechanosensitivity. Furthermore, experiments investigating the mechanisms of TRPV1 mechanosensitivity determined a role for TRPV1 in modulating purinergic responses on afferent nerves and ATP release from the urothelium. These mechanisms are thought to combine and underlie the decreased afferent nerve sensitivity to distension observed in TRPV1$^{-/-}$ knockout mice. This thesis also suggests that autocrine signalling of the urothelium through P2X and P2Y receptors may regulate intracellular calcium levels, an essential component of ATP release from the urothelium. Furthermore, a role for tachykinins in mediating mouse detrusor contraction acting through NK2 receptors was confirmed. A novel mechanism whereby stimulation of urothelial NK2 receptors was able to alter urothelial mediator release, and modify afferent nerve activity as a result of a change in detrusor function was also elucidated.

As modulation of bladder compliance and detrusor smooth muscle contraction during bladder distension was able to significantly alter afferent nerve discharge, these studies suggest that the major stimulus for afferent nerve output from the bladder is direct mechanical stretch of the bladder

wall, and that in healthy mice, a lesser component is attributable to secondary indirect mechanisms mediated via non-neuronal ATP from the urothelium.

Further research is necessary to determine the relative contribution of the two mechanosensitivity pathways in disease states, as there is significant evidence that a phenotypic switch towards an indirect mechanism of mechanosensation could underlie increased sensation and reflex bladder symptoms.

DECLARATION AND ADDENDUM

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research

This research represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this university or any other institution, except where due acknowledgment has been made.

Luke Grundy

April 2014

ACKNOWLEDGEMENTS

I would like to thank a great deal more people than can possibly be named within this section as I like to think that everyone who I have met during my PhD candidature has contributed to my growth as a person and therefore the completion of this thesis.

I would like to start by thanking my supervisors Professor Russ Chess-Williams and Dr Donna Sellers for their support over the last 4 years. I would also convey further thanks to Russ for initially offering me a chance to work in the laboratory here at Bond and the wonderful opportunity to study for my PhD, it has truly been the best experience I could have ever hoped for.

This thesis would not have been completed without the support of Bond University and NHMRC Australia who have provided me with the funds necessary to both carry out the work necessary and travel to both national and international conferences to present it. I would also like to thank all of the staff and students in the Faculty of Health Science and Medicine here at Bond University, and all of the staff in the lab at the University of Sheffield for unquestioningly accepting me into their research group during my time there. Particular mention should be made to my PhD colleagues who have provided valuable scientific discussion, and also invaluable non-scientific discussion during my time here. I would like to express my gratitude to Donna Daly in particular for her fantastic advice and providing her expert assistance in developing my scientific skills.

I could not have completed this PhD without the friends I have been lucky enough to find here on the Gold Coast, I thank you for all the good times, for showing me your country, and for listening to my presentations with no clue as to what they are about. A special mention must be made to my good friend Rob whom convinced me to undertake a PhD, I will be forever grateful.

I would like to acknowledge my girlfriend Ravi. For late night beach walks, for always making me smile, for her unwavering support, and for always understanding when I needed to work late and for putting up with me for the last three months.

These acknowledgements could not be complete without a mention of my family. To my Mum and Dad, who have always supported me in everything that I choose and pushed me to succeed, and to my brothers Joe and Rick for teaching me that there are no boundaries in life.

PUBLICATIONS

Abstracts as a result of this thesis

Grundy, L., Daly D, Chess-Williams R & Grundy D (2011) The role of TRPV1 in P2X mediated bladder afferent signalling. Proc. ASCEPT Annual meeting (Perth) Available online at http://ascept2011.eproceedings.com.au/papers/p183.pdf

Grundy, L., Chess-Williams R & Grundy, D. (2012) Primary mouse urothelial cell response to ATP is mediated by P2X but not TRPV1 receptors. Neurourology and Urodynamics: 31 (6): Pages: 1021-1022

Grundy, L., Daly, D.; Mansfield, K. Chess-Williams R. Sensory nerve responses to ATP are regulated by TRPV1 receptors. Neurourology and Urodynamics: 31 (6): Pages: 794-796

Grundy, L., Chess-Williams R (2012) Neurokinin A potentiates spontaneous and purinergic evoked smooth muscle contraction and bladder afferent activity responses via activation of mouse bladder urothelial and detrusor NK2 receptors. Proc. ASCEPT Annual meeting (Sydney) Available online at: http://www.ascept-apsa.com/wp-content/uploads/2012/07/ASCEPT-APSA-Oral-Abstracts-100-201.pdf

Grundy, L., Mills, K., Chess-Williams R & Grundy, D. (2013) Modulation of urothelial function and afferent sensitivity by Neurokinin A. Neurourology and Urodynamics: 32 (6) Pages 637-639

TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

1.1 ANATOMY OF THE BLADDER

The lower urinary tract (LUT) is composed of the urinary bladder and urethra which work in synchrony to provide temporary storage and effective voiding of urine delivered via the ureters from the kidneys.

The urinary bladder is a collapsible muscular organ with openings for the ureters and urethra (**Fig 1.1.1**) lined by a mucosal membrane on its luminal aspect and a serosal membrane on its outer aspect. The bladder stores urine between periods of emptying called micturition. In humans the bladder can hold up to a maximum of around 1000ml of urine if necessary, but a more modest 300-500ml represents a normal adult micturition volume. The bladder lies posterior to the pubic symphysis and when empty it is contained entirely within in the pelvis. The bladder is tethered to the pubic and pelvic bones by the pubovesical ligaments which help to maintain a stable position within the pelvis. The bladder is composed of functionally and histologically distinct regions.

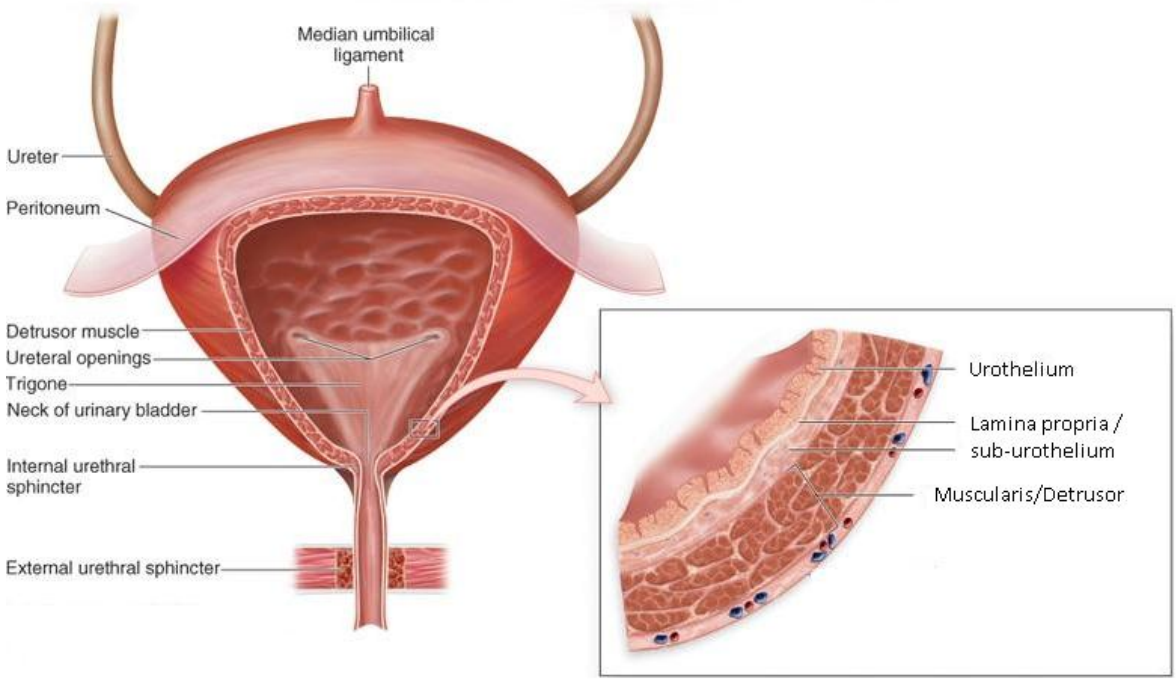

Fig 1.1.1 *Diagrammatic representation of the lower urinary tract and bladder wall of the human*. Modified from (Mckinley 2009).

Bladder histology

The bladder, as seen in **fig 1.1.2** is composed of numerous layers, the functions of which will be described in further detail below. The luminal side of the bladder is composed of the urothelium which in turn is composed of stratified epithelial cells arranged into three layers of different cell types composing from the apical side: umbrella cells, intermediate cells and basal cells (Lewis 2000). Beneath the urothelium is the lamina propria/sub-urothelium which is composed of extensive innervations of afferent nerve fibers, dense vasculature and a network of interstitial cells. Together, the urothelium and suburothelium are often referred to as the mucosa despite a lack of mucous secreting cells. Below the suburothelium is the muscular layer composed of three layers: a large circular muscle layer which is enclosed by outer and inner longitudinal muscle. The function of the bladder relies on coordinated activity between all the layers of the bladder, and each of these will now be described in more detail.

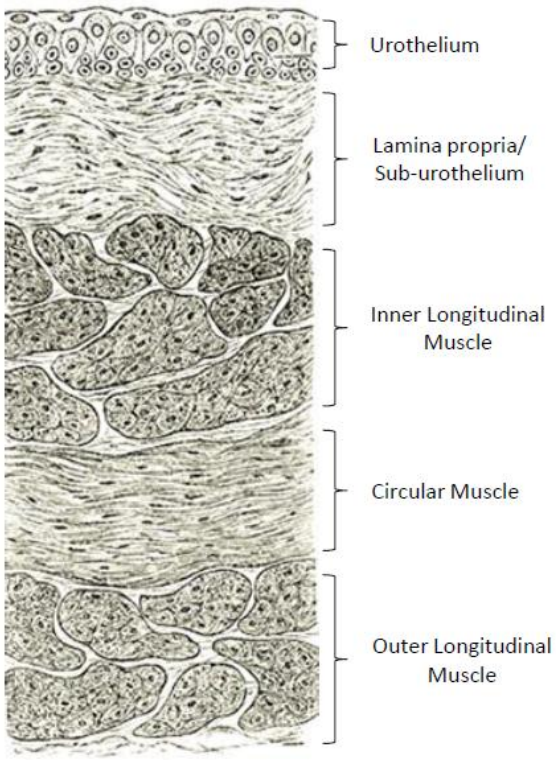

***Fig 1.1.2**. Cross section of the bladder wall, adapted from (Martini 2001)*

1.2 THE BLADDER WALL: STRUCTURE AND FUNCTION

Urothelium

Structure

The urothelium, when viewed as a cross section is arranged into three layers of different cell types composing umbrella cells, intermediate cells and basal cells typically 5-7 cells thick (Lewis 2000). Basal cells with a diameter of 5-10μm lie in a single layer attached to a basement membrane, intermediate cells are pyriform in shape and vary in size around 20μm but can be arranged in a layer that is several cells deep. The superficial apical umbrella cells are large hexagonal shaped cells and vary in length between 25 – 250μm based on the degree of bladder stretch (Apodaca 2004).

Barrier function

The primary function of the urothelium is to serve as a passive diffusion barrier, preventing urine contents from entering the blood. There is now compelling evidence the urothelium also performs an important role in the detection of mechanical, chemical and thermal stimuli essential to normal storage and voiding of urine (Birder 2006; Yoshimura, Kaiho et al. 2008), which will later be discussed in detail. The ability of the urothelium to function as an effective barrier is provided through a number of specific properties of the umbrella cells including high resistance tight junctions (Lewis, Berg et al. 1995), which limit the movement of ions and solutes between cells. The luminal surface of umbrella cells has a unique lipid protein membrane structure composed of plaque (uroplakins) and hinge regions with a glycosaminoglycan (GAG) layer on the luminal membrane (Lewis 2000) which reduces the permeability of the urothelium to small molecules such as water, urea and protons.

Accommodation to stretch

An essential feature of the urothelium is the ability to maintain a barrier function during large increases in surface area as the bladder is distended. This is considered to involve two phases; with the initial increases in surface area being accommodated by microscopic adjustments of the structure as the intermediate and basal cell layers are pushed laterally in an unfolding of the epithelium from distinct rugae when empty (Minsky and Chlapowski 1978). A second mechanism involves the generation of an expanded umbrella cell membrane from a roughly cuboidal shape to a

more flat and squamous morphology. This is due to calcium and cAMP dependent pressure induced exocytosis of fusiform/discoid vesicles from within the cells (Truschel, Wang et al. 2002; Wang, Truschel et al. 2003). The process of exocytosis encompasses a number of steps which combine to accomplish cell surface supply and secretion of biologically relevant molecules. Vesicles, usually preformed, are trafficked over short distances from within the cell cytoplasm to the apical cell membrane, where they first tether, dock and then fuse with the plasma membrane via the association of the SNARE complex. The contents of the vesicle are expelled into the exterior whilst the vesicle membrane is incorporated into the plasma membrane of the cell, increasing its surface area. This process allows umbrella cells to change shape with a dramatic increase in the apical surface area. It has been shown that ATP is released from the urothelium in response to stretch (Birder, Nakamura et al. 2002; Sadananda, Shang et al. 2009; Dunning-Davies, Fry et al. 2013) and purinergic receptors have been recently implicated in mediating vesicle exocytosis via Ca^{2+} mobilisation or influx (Wang, Lee et al. 2005). Post voiding, the membrane recycles into endosomes and the resting shape is recovered.

Sensory function of the urothelium

The role of the urothelium, as briefly mentioned above, extends beyond a simple barrier function and has in recent years been implicated in an important role for visceral sensation. The urothelium, which is innervated by afferent and efferent nerves, has been described as having neuronal properties due to its ability to respond to an array of chemical messengers often reserved for neurotransmission including adenosine triphosphate (ATP), and acetylcholine (ACh). Urothelial cells achieve this function via the expression of functional ion channels and receptors normally present on nerve endings including purinergic, transient receptor potential (TRP), muscarinic, and nicotinic (Birder, Kanai et al. 2001; Stein, Santos et al. 2004; Beckel, Kanai et al. 2005; Mansfield, Liu et al. 2005; Zarghooni, Wunsch et al. 2007; Streng, Axelsson et al. 2008). The close association of bladder afferent nerves to the urothelium, and the above mentioned neuronal properties of these cells have supported a theory that urothelial-neural communications occurs and together forms a sensory structure. It is also probable that interstitial cells present within the suburothelium (fig 1.1.2) contribute to this sensory structure and their roles will also be discussed. Activation of capsaicin sensitive primary afferents (CSPANS) has been shown to induce the release of neuropeptides (Maggi, Patacchini et al. 1991; Lecci, Giuliani et al. 1997) which have the capacity to influence the urothelium and interstitial cells. Stretch and mechanical stimulation of the urothelium in-situ or cultured urothelial cells has been shown to stimulate the release of a number of neurotransmitters including

NO, ATP, Prostaglandins (PGs), and ACh (Birder, Nakamura et al. 2002; Hanna-Mitchell, Beckel et al. 2007; Yokoyama, Tanaka et al. 2011; Collins, Daly et al. 2013). The receptors for these neurotransmitters and PGs are located on the underlying structures of the bladder, including the sensory afferent nerves, detrusor smooth muscle, and interstitial cells, and it is proposed that these neurotransmitters may act to modulate bladder sensory output to the spinal cord.

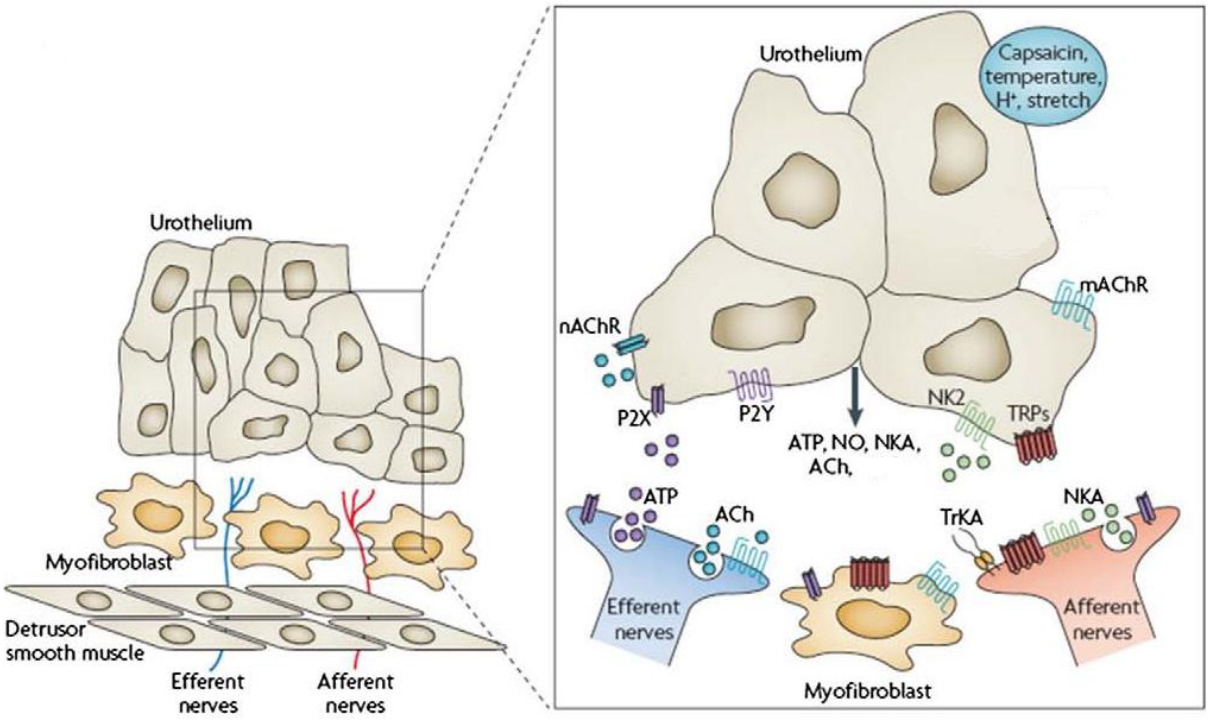

Fig 1.2.1 *Diagrammatic representation of the afferent and efferent innervations of the urinary bladder wall, and a hypothetical model of interactions between neuronal and non-neuronal cells within the urothelium/sub-urothelium. A number of mediators are released within the urinary bladder wall and have the ability to act in either an autocrine or paracrine manner to alter neuronal excitability. Abbreviations: ACh, acetylcholine; ATP, adenosine triphosphate; H^+, proton, mAChR, muscarinic receptor; NKA, neurokinin A; NK2, tachykinin 2 receptor; nAChR, nicotinic receptor; NO, nitric oxide; P2X and P2Y; purinergic receptors; trkA, receptor tyrosine kinase A, high affinity receptor for nerve growth factor; TRPs, transient potential channels. Modified from Birder (2010).*

Interstitial cells

There is controversy surrounding the nomenclature and organisation of the cells which comprise the lamina propria and this is caused by a lack of specific markers and some unusual structural and functional characteristics. The electrically active cells of the lamina propria change both in

morphology and ultrastructure as they appear closer to the detrusor muscle layer (Wiseman, Fowler et al. 2003). As morphological differentiation has not been made, in this thesis they shall be encompassed by the term interstitial cells (IC).

The Interstitial Cells of Cajal (ICC) in the gut act as pacemaker cells, co-ordinating peristaltic contractions and facilitating neuromuscular transmission (Sanders and Ward 2007). These spindle-shaped cells with multiple thin branching processes and expressing markers such as c-kit, and connexin-43 (McCloskey 2010) have been identified in the bladder where they are found to form two discrete populations of ICs; one such population forms a distinct plexus in the sub-urothelium where they are found in close association with afferent nerves (Sui, Rothery et al. 2002; Wiseman, Fowler et al. 2003; Fry, Sui et al. 2007). The second population of ICs are found in around the smooth muscle layers and are also found to make close structural connections with nerves (Davidson and McCloskey 2005; McCloskey, Anderson et al. 2009).

A specific role for ICs in the bladder has yet to be established but a number of theories have been proposed. It has been anticipated that the population of ICs found within detrusor smooth muscle may contribute to the spontaneous myocyte activity of the detrusor smooth muscle; although the mechanism by which this would occur is still controversial. Gap junctions provide a mechanism for direct cell-cell electrical communication and it is thought that the ICs may act as a functional synctium linked to the detrusor smooth muscle, influencing contraction (Wiseman, Fowler et al. 2003). However, despite the electrical activity of ICs, characterised by spontaneous membrane current fluctuations which are activated by a rise in intracellular Ca^{2+} and dependent on Cl^- currents (Fry, Sui et al. 2007), the calcium transients from detrusor myocytes appear to be independent of IC function (Hashitani, Brading et al. 2004; Sui, Wu et al. 2004).

The extensive gap junctions which couple ICs, allow them to act as a unit to propagate electrical activity throughout the sub-urothelial layer, and they are proposed to integrate signals from the urothelium with electrical and chemical information from efferent nerves. In providing this service, ICs are in a key position in close proximity to sensory afferents and thus have the potential to influence the excitability of nerve terminals (Wiseman, Fowler et al. 2003; Sui, Wu et al. 2004), and influence afferent transduction. ATP acting on purinergic receptors (Cheng, Scigalla et al. 2011), and ACh via muscarinic receptors (Johnston, Carson et al. 2008) have been shown to initiate intracellular Ca^{2+} transients in ICs, and due to the known release of these neurotransmitters from the urothelium and the propinquity of urothelial cells to ICs, it is likely that a functional interaction occurs.

Detrusor smooth muscle

The detrusor smooth muscle consists of a layer of circular muscle which is enclosed by outer and inner longitudinal muscle layers. Individual detrusor smooth muscle cells are long, spindle shaped cells arranged in bundles surrounded by collagen. The smooth muscle cells contain actin and myosin filaments, along with cytoskeletal intermediate filaments and dense bodies containing the microfilament protein actinin (Andersson and Arner 2004).

Detrusor smooth muscle cells elicit an action potential from a resting potential of -50 to -60mV (Sui, Wu et al. 2001). The depolarisation phase is mediated via an inward current of Ca^{2+} via L-type Ca^{2+} channels (Kajioka, Nakayama et al. 2002). ATP-sensitive K^+ channels have also been shown to influence bladder contractility and cause hyperpolarisation (Bonev and Nelson 1993), while calcium activated K^+ channels play a role in the maintenance of the resting potential and in repolarisation of the detrusor muscle cell membrane (Montgomery and Fry 1992).

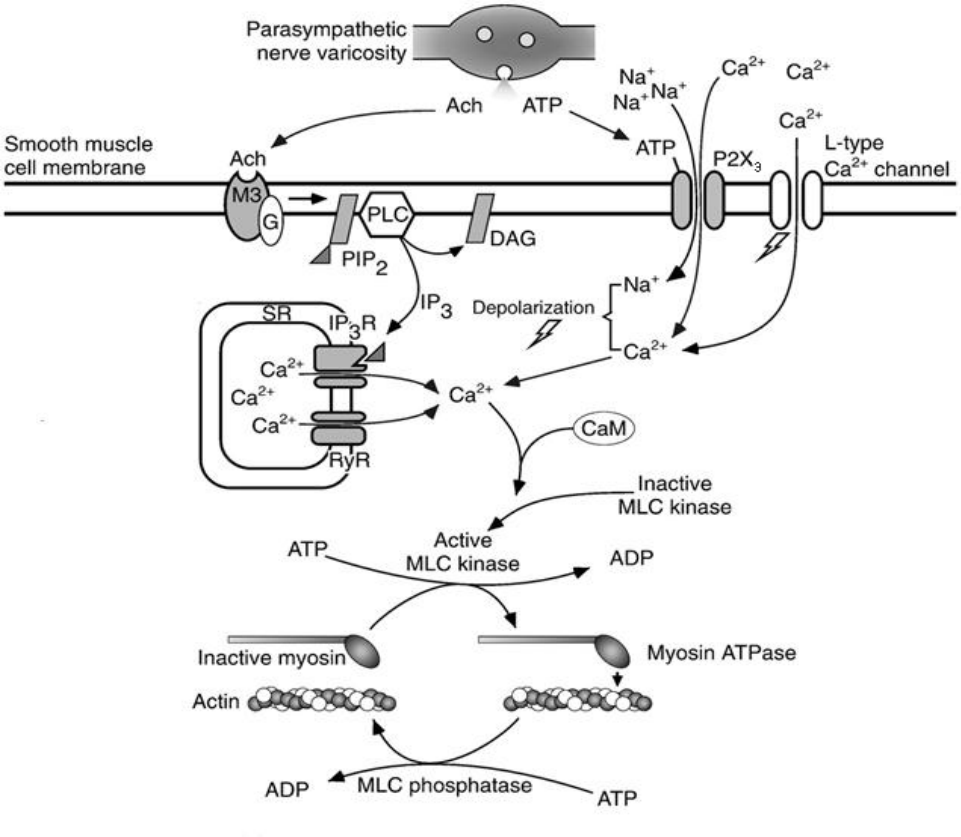

Fig 1.2.2 *An example of one of the important intracellular signalling pathways initiated by acetylcholine binding to M_3 receptors and ATP binding to $P2X_3$ receptors in determining detrusor smooth muscle cell contraction. Abbreviations: Ca^{2+}, calcium; CaM, calmodulin; DAG, diacylglycerol; IP_3, inositol trisphosphate; MLC, myosin light chain; PIP_2, phospholipid; PLC, phospholipase-C; RyR, ryanodine receptor; SR, sarcoplasmic reticulum. Modified from Fry et al (2010).*

ACh is released from parasympathetic nerves to initiate contraction of the detrusor whilst noradrenaline released from sympathetic neurons cause relaxation. Neurotransmitter release from the parasympathetic nerves innervating the detrusor, along with local release of transmitters from the urothelium in response to stretch, activates receptors on the detrusor smooth muscle membrane. Ligand receptor binding on the cell membrane results in an increase in intracellular Ca^{2+}. This is either from extracellular sources, via L-type Ca^{2+} channels (Kajioka, Nakayama et al. 2002), or intracellular; via Ca^{2+} induced Ca^{2+} release by ryanodine receptor activation on the sarcoplasmic reticulum (Hashitani and Brading 2003), or G-protein coupled IP_3 receptor mediated release (Fry, Meng et al. 2010). Ca^{2+} binds to calmodulin in the cytoplasm which is then able to activate myosin light chain kinase (MLCK). Activated MLCK phosphorylates the myosin light chain which allows it to interact between actin and myosin to cause contraction (**fig 1.2.2**) (Fry, Skennerton et al. 2002).

There is evidence for the presence of a number of receptors on the detrusor smooth muscle which are thought to have modulator effects on the primarily muscarinic and purinergic components of detrusor contraction which will be discussed in further detail below.

1.3 NEURAL INNERVATIONS OF THE BLADDER

Micturition pathway

The function of the urinary bladder is to effectively store and eliminate urine, and is dependent on the integration of a complex neural network comprising sympathetic, parasympathetic and somatic afferent and efferent pathways from the periphery, spinal cord, brainstem, and cortical regions under both voluntary and involuntary control.

Efferent pathways

The efferent parasympathetic pathway, originating from the sacral spinal cord provides the major excitatory input to the urinary bladder.

Preganglionic cholinergic neurons located in the intermediolateral region of the sacral spinal cord (S2 – S4, (laminae v - vii)) project axons as the pelvic nerve to synapse in the pelvic ganglia as well as directly into the intramural autonomic ganglia in the bladder wall (Yoshimura, Kaiho et al. 2008). Postganglionic neuronal activation is mediated via ACh acting on nicotinic ACh receptors (nAChR) (Wang, Orr-Urtreger et al. 2002) but can be influenced by many other receptors including those for noradrenaline, the ionotropic P2X and metabotropic P2Y receptors for ATP, as well as various peptides (Keast, Kawatani et al. 1990; Tompkins, Girard et al. 2010). Postganglionic axons continue as the pelvic nerve and terminate in the detrusor smooth muscle. Terminal regions of parasympathetic postganglionic efferents have vesicle filled varicosities capable of releasing neurotransmitter to stimulate contraction via the predominant actions of ACh as well as other non-adrenergic non-cholinergic (NANC) neurotransmitters (de Groat 2006).

Contraction of the detrusor by ACh is mediated by atropine sensitive M3 receptors despite the predominance of M2 receptors in this tissue (Chess-Williams, Chapple et al. 2001; Matsui, Motomura et al. 2002). There is an atropine resistant component of excitatory transmission which has been shown to be mediated by ATP acting on ionotropic P2X purinoceptors (Burnstock 2001) and a number of co-released peptides including vasointestinal peptide (VIP) and neuropeptide Y (NPY) which may be inhibitory or excitatory (Andersson and Wein 2004). Although the contribution of P2X receptor activation to detrusor contraction appears to be minor in healthy human tissue (Ford, Gever et al. 2006), it has been shown that this contribution can dramatically increase in

certain bladder pathologies (Bayliss, Wu et al. 1999; O'Reilly, Kosaka et al. 2002; Brady, Apostolidis et al. 2004) as well as with age (Yoshida, Miyamae et al. 2004).

In conjunction with the excitatory input to the detrusor smooth muscle, postganglionic parasympathetic efferents have been shown to provide an inhibitory input to the urethral smooth muscle in the form of nitric oxide (Andersson and Arner 2004). The efferent sympathetic pathway, originating in the thoracolumbar section of the spinal cord (T11-L2) passes to the lumbosacral sympathetic chain ganglia and the pelvic plexus, or to short adrenergic neurons in the bladder and urethra (de Groat 1997). Sympathetic postganglionic nerves release noradrenaline and provide the major excitatory input to the urethra via α_1 adrenoceptors and inhibitory input to the detrusor via β adrenoceptors (de Groat 2006).

Preganglionic sympathetic neurons pass to the lumbosacral paravertebral ganglia and the pelvic plexus to synapse in the inferior mesenteric ganglia (Birder 2006) via the actions of ACh on nAChR in a similar manner to parasympathetic fibers in the pelvic ganglia. Postganglionic axons travel as the hypogastric nerve to three major targets where noradrenaline is the principal transmitter released. Noradrenaline acts via α_1-adrenoceptors on the bladder neck and urethra to stimulate contraction (Anderson 1993) whilst also acting via β_2 and β_3-adrenoceptors on the detrusor to induce relaxation (Anderson 1993; Igawa, Yamazaki et al. 1999). Furthermore, it has also been shown that sympathetic fibers project to the pelvic ganglia where release of noradrenaline provides inhibition of excitatory parasympathetic nerve activity supplying the detrusor (de Groat 2001).

Efferent innervations of the striated muscles of the external urethral sphincter (EUS) is provided by somatic nerves originating from Onuf's nucleus in the lateral ventral horn of the S2-S4 sacral spinal cord (Thor, Morgan et al. 1989). Sphincter motoneurons are carried in the pudendal nerve and initiate contraction of the EUS via release of ACh onto nAchR (de Groat 2001; de Groat 2006).

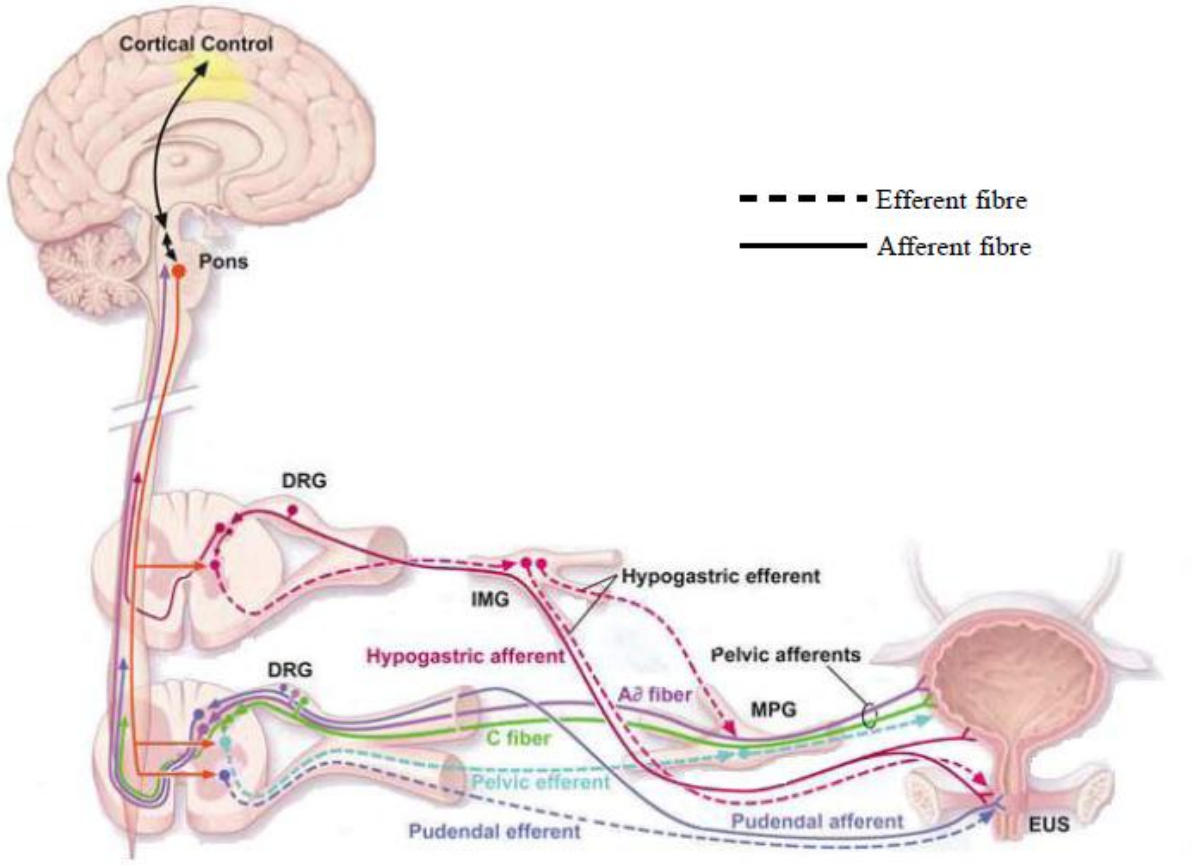

Fig 1.3.1 *Diagrammatical representation of the neural circuits maintaining continence and stimulating micturition in the bladder. Pelvic nerves convey the major afferent innervations of the bladder via Aδ- and C-fiber afferents to the lumbo-sacral region of the spinal cord, whilst a smaller component of bladder afferent innervations travel in the hypogastric nerve to the thoracolumbar region of the spinal cord. Parasympathetic and sympathetic efferents travel in the pelvic and hypogastric nerve respectively. Sacral somatic afferent and efferent innervations to the external urethral sphincter are via pudendal nerves. Abbreviations: DRG, dorsal root ganglion; EUS, external urethral sphincter; IMG, inferior mesenteric ganglion; MPG, pelvic ganglion. Taken from Ford et al (2006)*

Afferent Pathways

Sensory information regarding the state of the lower urinary tract is carried in small myelinated Aδ and unmyelinated C-fibers via the pelvic, pudendal and hypogastric nerves to the lumbosacral spinal cord. Pelvic and pudendal nerves predominantly have their cell bodies within the dorsal root ganglion (DRG) at the level of the sacral spinal cord (S1-S4) whilst hypogastric nerves project via DRGs to the thoracolumbar segments (T10-L2) of the spinal cord. Ascending afferent projections travel via the lateral and dorsal funiculi and synapse in a number of nuclei in the midbrain and brainstem including the periaqueductal grey matter (PAG) (de Groat 1997). These neurons convey

information to the pontine micturition centre to initiate the micturition reflex (Blok, Willemsen et al. 1997).

Bladder afferents projecting via parasympathetic pathways are essential for maintaining normal bladder function. They are found in distinct populations innervating the detrusor smooth muscle, in close proximity to blood vessels, in the sub-urothelium and also innervating the urothelium itself (Sharkey, Williams et al. 1983; Su, Wharton et al. 1986). The distribution of bladder afferent axons has been studied in a number of species and afferent nerves have been classified based primarily on conduction velocities, neuropeptide content and their sensitivity to mechanical stimuli: and are either high or low threshold. The afferent nerves innervating the urinary tract are composed of unmyelinated (C-fiber) axons which comprise approximately 60-70% of total afferent fibers, with the remainder consisting of small myelinated (Aδ) fibers (Vera and Nadelhaft 1990; Andersson 2002). The majority of bladder dorsal root ganglion (DRG) neurons also contain multiple neuropeptides, including calcitonin gene-related peptide (CGRP), substance P, and vasointestinal peptide (VIP) (Keast and de Groat 1992; Smet, Moore et al. 1997) and a significant proportion of these are capsaicin sensitive (Avelino and Cruz 2000; Avelino, Cruz et al. 2002). Mechanoreceptive afferents can also be sub-classified into a larger proportion of low threshold (approx 80%) and fewer high threshold (approx 20%) fibers (Sengupta and Gebhart 1994; Shea, Cai et al. 2000). Denervation studies have shown that bladder afferent innervations are bilateral with an intermingling of axons supplying both contralateral and ipsilateral regions (Gabella and Davis 1998).

The distribution of axons within the bladder is variable and dependent on the region. Afferent innervations of the detrusor is relatively diffuse, but the density of these innervations is uniform throughout the bladder (Gabella and Davis 1998; Birder, de Groat et al. 2010). The sub-urothelium contains the highest density of axons in the bladder which form a plexus lying just above the basal surface of the urothelium. The sub-urothelial plexus is thickest at the bladder neck (Andersson 2002) and adjacent urethra, and becomes more diffuse in adjacent regions (Smet, Edyvane et al. 1996; Gabella and Davis 1998). There is also a proportion of axons which penetrate the urothelial layer and are thought to branch from the sub-epithelial plexus. These fibers, as with those innervating the detrusor, are considered to be direct mechanosensors. The afferent nerves of the suburothelium are proposed to have a less direct influence on mechanosensitivity but respond to mediators released from the urothelium. These afferent are also proposed to have an efferent function related to the release of neuropeptides stored in their peripheral varicosities which are released upon excitation (Maggi, Patacchini et al. 1991; Smet, Edyvane et al. 1996; Lecci, Giuliani et al. 1997) and are believed

to perform a local regulatory role on the underlying structures of the bladder including the modulation of afferent nerve excitability (Sculptoreanu and de Groat 2007).

Classification of sensory neurons within the bladder

As briefly described, the sensory afferent nerves innervating the bladder belong to different functional populations which are thought to underlie their distinct stimulus-response profiles. It has been shown that the most important afferents for input into the micturition reflex are those travelling in the pelvic nerves to the sacral spinal cord. This is the branch of the micturition pathway which will be the focus of this thesis.

Pelvic afferents consist of small myelinated Aδ fibers with a conduction velocity of $\approx 11 ms^{-1}$, and unmyelinated C fibers with a conduction velocity of $\leq 2 ms^{-1}$. Electrophysiology studies have shown that bladder afferents respond to bladder distension (Vlaskovska, Kasakov et al. 2001; Rong, Spyer et al. 2002; Daly, Rong et al. 2007) or active contraction. Within these afferents, there was found to be a subset that responds to distension but not contraction and were dubbed 'volume' receptors (Morrison 1999). A number of studies have classified these afferents functionally by their activation threshold, and a distinction between a population of low, and high threshold afferents has been made based on their response to bladder distension (Rong, Spyer et al. 2002; Zagorodnyuk, Costa et al. 2006; Daly, Rong et al. 2007). Although there are inter-experimental differences in the precise definitions, low threshold afferents are activated at low intravesical pressures from 3-15mmHg and show an increased afferent nerve response as pressures increases up to a point following which, the response plateaus (Shea, Cai et al. 2000; Rong, Spyer et al. 2002; Daly, Rong et al. 2007). High threshold afferents also respond to an increase in bladder pressures but their activation is initiated at a higher threshold and increases past physiological voiding pressures. Populations of Aδ- and C-fibers respond to both high and low thresholds suggesting there is no correlation between conduction velocity and afferent subtype (Sengupta and Gebhart 1994; Shea, Cai et al. 2000).

Afferent nerve terminals located in the detrusor muscle layers, urothelium and sub-urothelium all convey vital information regarding the state of the bladder to the CNS and in this way, control the micturition reflex. Traditionally, afferent nerves have been broadly classified as either mechanosensitive or chemosensitive fibers. Thus, they either respond to dynamic stretch due to physical alterations in the bladder wall, or the actions of chemical stimuli binding to receptors on the afferent nerve membrane. It has now been possible to further classify these subtypes based on their receptive field as well as the stimuli they respond to. Two research groups have recently proposed

different nomenclature for what appears to be essentially a description of the same subtypes of afferents and will be discussed in further detail here.

1. Muscle mechanoreceptors

A consensus that has formed is the location of mechanosensitive afferents which innervate the detrusor smooth muscle layers of the bladder. They are powerfully activated by stretch (Zagorodnyuk, Costa et al. 2006; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008) but not by stroking with von-frey hairs or chemical compounds such as ATP or capsaicin. Thus they are considered pure mechanoreceptors. The proportion of afferents which are muscle mechanoreceptors is species dependent, in guinea pig they contributed 15% of total recorded, whereas in mouse, that number was around 45% for mixed pelvic and lumbosacral afferents (Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). In physiological terms these mechanoreceptors are believed to respond to bladder distension by sensing intravesical pressure, and by acting as 'in-series' tension receptors previously described (Habler, Janig et al. 1993; Shea, Cai et al. 2000). It has previously been shown that afferent activity maintains a precise relationship with the change in tension of the receptive field of these afferents (Downie and Armour 1992). Muscle mechanoreceptors are composed of both low and high threshold components and it has also been shown that they can be sensitised by the administration of an inflammatory soup (Xu and Gebhart 2008). Removal of the urothelium has no effect on stretch induced firing, which suggests that the receptive field for these muscle mechanoreceptors is just that, contained within the muscle.

2. Muscular/urothelial afferents

The nomenclature of the muscular/urothelial mechanoreceptors is related to their location in the bladder, innervating both the detrusor smooth muscle and the sub-urothelium. Considered to be similar to the previously reported muscular/mucosa afferents of the pelvic nerve in the distal gastrointestinal tract (Brierley, Jones Iii et al. 2004). Unlike muscle mechanoreceptors, removal of the urothelium significantly reduces distension induced afferent firing. They respond to both mechanical stretch and light von-frey hair stroking and contribute around 30% of the total afferents in the bladder wall (Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). Activation is possible by ATP (Zagorodnyuk, Gibbins et al. 2007), and thus their location close to the urothelium suggests that they might respond to ATP released by urothelial distension (Sadananda, Shang et al. 2009; Sadananda, Kao et al. 2012; Collins, Daly et al. 2013). Considered to respond to a lower threshold of

stimuli than muscle mechanoreceptors, they are responsive to bladder contractions and most probably act as a sub-set of in-series tension receptors (Häbler, Jänig et al. 1993; Shea, Cai et al. 2000) able to coordinate stretch across the various layers of the bladder wall.

3. Urothelial afferents

A subset of urothelial afferents, distinct from muscular/urothelial afferents that make up between 10-30% of the total afferent discharge was also observed in mouse and guinea pig (Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). These urothelial afferents are distension insensitive, but activated by mucosal stroking with light von-frey hairs, and can also be activated by various chemical stimuli (Zagorodnyuk, Costa et al. 2006; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008; Zagorodnyuk, Brookes et al. 2009). A similar fraction of distension-insensitive (mucosal) afferents have been identified in the mouse colon (Brierley, Jones Iii et al. 2004). As with those described in the bladder, despite failing to respond to mechanical stretch/distension, these mucosal afferents respond to a range of other stimuli including mucosal stroking and probing. It is proposed that these afferents may become distension sensitive during inflammation or insult to the bladder and may transmit pain sensations. A second class of urothelial mechanoreceptor was also identified by zagorodynuk et al, in which these afferents, aside from being insensitive to distension, also fail to respond to chemical stimuli. This division of urothelial afferents shares a number of characteristics with distension-insensitive afferents previously termed silent nociceptors (Jänig and Koltzenburg 1990; Habler, Janig et al. 1993) and are described in further detail below.

4. Silent Afferents

There are a proportion of sensory fibers innervating the viscera which do not respond to high intensity mechanical or noxious chemical stimuli. In all recent experiments aimed at elucidating the subsets of afferent neurons which mediate afferent nerve discharge, there has been a proportion of afferents which are unresponsive to mechanical stimulation, and a proportion of these which are unresponsive to chemical stimuli (Shea, Cai et al. 2000; Rong, Spyer et al. 2002; Daly, Rong et al. 2007; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). These receptors, as outlined above, have been termed ‘silent’ nociceptors. Studies have also shown that sensitisation of previously unresponsive afferents is possible (Häbler, Jänig et al. 1990; Rong, Spyer et al. 2002) and thus there is evidence for a population of pelvic afferents which under normal conditions are unresponsive to a full range of stimuli, and become activated only following acute irritation or inflammation. It is worth mentioning that the ability of inflammatory mediators to induce afferent sensitisation is not limited

to ‘silent’ afferents but is also seen in both low and high threshold mechanoreceptors (Rong, Spyer et al. 2002; Xu and Gebhart 2008).

1.4 MECHANISMS OF MECHANOSENSITIVITY

Direct mechanotransduction

There is a large proportion of bladder mechanosensation which is directly coupled to the mechanical forces exerted on the detrusor smooth muscle during distension and is evident by the relationship of bladder volume, pressure, or tension with afferent nerve discharge. Direct mechanotransduction has been studied in a number of physiological systems and cell types, and numerous molecules and sub-cellular structures have been shown to mediate mechanical mechanisms into biochemical activity. The concept of direct mechanotransduction in visceral afferent nerves is determined by direct gating of ion channels in response to mechanical force. For this to occur, an ion channel structure capable of responding to a mechanical stimulus must be present at the location where a mechanical stimuli is detected (Gillespie and Walker 2001). Currently, the molecular identity of such a channel is elusive, but a number of candidates including the epithelial sodium channel (ENaC)/Acid sensing ion channel (ASIC)/degenerin Na^+ channels, and TRP channels (Sun, Li et al. 2010) are proposed, which have been shown to modulate the detection of mechanical stimuli in a number of species (Gillespie and Walker 2001; Strassmaier and Gillespie 2002; Goodman, Lumpkin et al. 2004). A recent review of direct mechanotransduction in the gastrointestinal tract has explored this area (Brierley 2010) and concluded that the detection of mechanical stimuli by visceral afferents may be fundamentally different to somatic systems.

The generation of a graded receptor potential in afferent neurons in response to stretch requires the gating of a channel, which in the case of physiological bladder filling must be sensitive to incremental increases in bladder pressure/muscle stretch and can be rapidly activated to compensate for these changes (Brierley 2010), and thus influence the frequency of the action potentials generated. The first major hypothesis for direct mechanotransduction relies on the direct distortion of the ion channel structure, which can be the result of a change in membrane bilayer tension, a 'tugging' of the ion channel, physically opening the pore (Hamill and Martinac 2001). The second major hypothesis is that of a tethered channel, in which the proteins of the extracellular matrix or intracellular cytoskeleton are linked to the stretch activated domains of the ion channel and thus act as intermediates to alter tension throughout the entire cell membrane, which results in an opening of the channel (Gillespie and Walker 2001; Christensen and Corey 2007).

One of the main stumbling blocks for determining the methods of direct mechanotransduction in the bladder, as described previously, is the heterogeneity of the afferents innervating the different

layers of the bladder, and the likelihood of the transduction mechanisms being conserved between them.

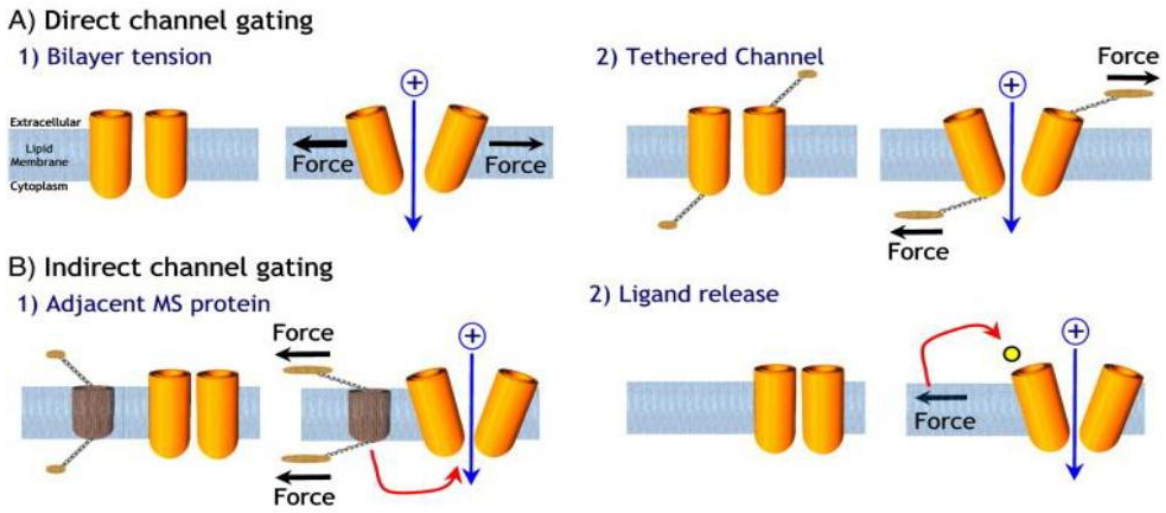

Fig 1.4.1 *Proposed models by which a putative mechanotransduction channel is gated by mechanical stimuli (Brierley 2010).*

Numerous studies have been completed which have elucidated a role for ASIC1, ASIC2, ASIC3, TRPV1, TRPV4, and TRPA1 channels in mediating mechanosensitivity in the gastrointestinal tract (Brierley 2010). However, as many of these channels also contribute to overall afferent sensitivity as well as possible roles in other structures of the bladder wall, including the mediator release from the urothelium, determining a direct mechanism of action is a particularly gruelling task.

Indirect mechanotransduction

As described above, there are subsets of afferent neurons which are distension insensitive, but express chemoreceptors able to respond to a number of chemical agents, including ATP. An indirect mechanism for mechanotransduction involving the interaction of afferent nerves with factors released from the surrounding tissues has been postulated. The main pathways thought to underlie this, which have been mentioned above, are the release of mediators from the urothelium in response to mechanical stretch, the release of neuropeptides from the terminal varicosities of afferent nerves, and the ability of interstitial cells to communicate electrically with afferent nerves. ATP and ACh are released from the urothelium in response to stretch (Ferguson, Kennedy et al. 1997; Hanna-Mitchell, Beckel et al. 2007; Dunning-Davies, Fry et al. 2013) and sub-urothelial afferent nerves express purinergic receptors which are stimulated by ATP, including the $P2X_3$ ionotropic receptor (Namasivayam, Eardley et al. 1999; Rong, Spyer et al. 2002; Cockayne, Dunn et al. 2005).

This mechanism has undergone intense research and is considered to be an important mechanism of mechanotransduction in the bladder as $P2X_3$ knockout mice exhibit significant bladder hyporeflexia (Cockayne, Hamilton et al. 2000; Vlaskovska, Kasakov et al. 2001). Another possible role for urothelially released ATP is the activation of the underlying interstitial cell network which has also been shown to express both P2X and P2Y purinoceptors (Sui, Wu et al. 2006; Li, Xue et al. 2013). In this model, ATP released from the urothelium is able to induce an exaggerated electrical current in interstitial cells, which are able to couple to afferent neurons and influence their excitability.

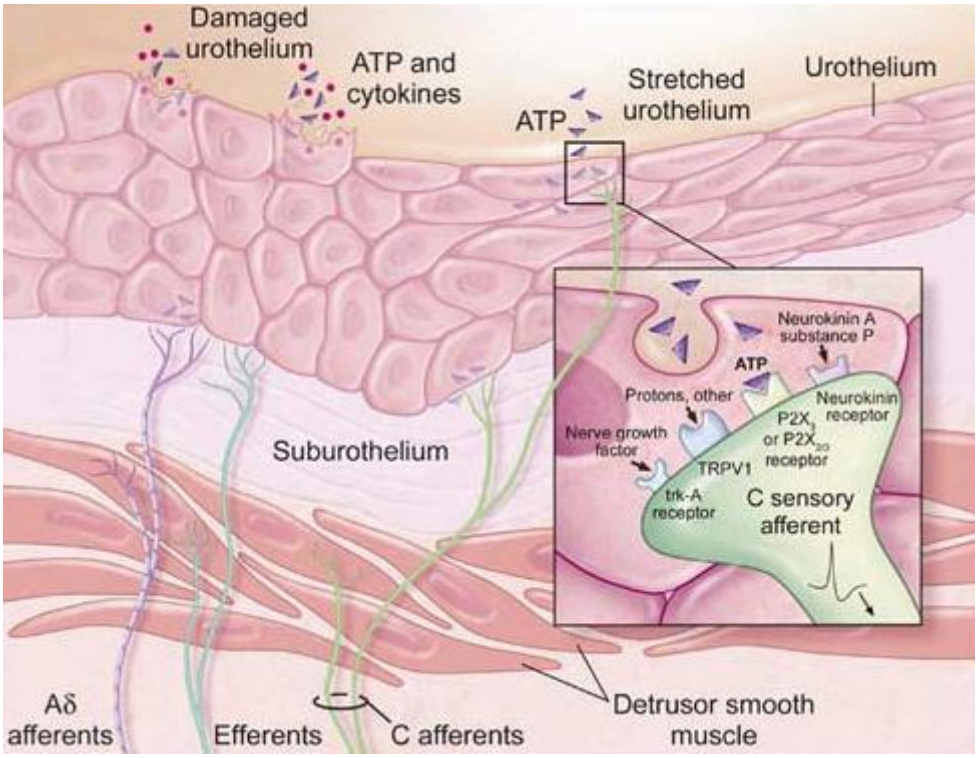

Fig 1.4.2 *Schematic diagram showing a proposed indirect mechanotransduction role for urothelial ATP. Bladder distension causes the release of ATP from the urothelium and ATP acts on purinergic receptors on the terminal varicosities of bladder afferents, where it may convey mechanosensory information to the central nervous system. Taken from Ford et al (2006)*

The ability of urothelially released ATP to influence mechanotransduction has been disputed by Zagorodyuk and colleagues. They have suggested that the onset of stretch induced afferent discharge in the guinea pig rectum is so fast (≈5ms) that it is likely due to a direct mechanism (Zagorodnyuk, Lynn et al. 2005). They have also shown that pharmacological blockade of P2X receptors or the use of calcium free medium to prevent exocytotic release of transmitters had no effect on mechanotransducion in guinea pig bladder (Zagorodnyuk, Costa et al. 2006). Removal of the urothelium was also shown to have no effect on stretch induced afferent discharge

(Zagorodnyuk, Gibbins et al. 2007). In rebuttal to these claims however, the exocytotic release of ATP from the urothelium has been shown to increase following removal of extracellular calcium (Matsumoto-Miyai, Kagase et al. 2009), and the stretch sensitive afferents from which they record are not those which are thought to depend on this secondary, indirect mechanotransduction mechanism.

As such, this pathway is still an unknown, and this thesis will attempt to clarify some of these issues.

1.5 THE MICTURITION REFLEX

Storage phase

The effective storage of urine is provided through spinal reflexes incorporating parasympathetic, sympathetic and somatic afferents from the bladder and urethral sphincters. During urine storage; discharge from stretch sensitive afferent nerves innervating the detrusor smooth muscle is low. This results in parasympathetic and sympathetic reflexes that aid bladder filling and facilitate continence by contracting the internal urethral sphincter (Roberts 2008). The bladder to external urethral sphincter reflex, also termed the 'guarding reflex', is a result of bladder afferent activation of pudendal motoneurons in the sacral spinal cord which stimulate external urethral sphincter contractions. The guarding reflex is also under supraspinal regulation, with the pontine storage centre in the dorsolateral pons providing descending input which activates pudendal motoneurons and increases urethral resistance (de Groat 2006; Yoshimura, Kaiho et al. 2008). There is also evidence for reciprocal pudendal afferent inhibition of parasympathetic efferent firing by suppression of spinal interneuron activation as well as inhibition of pelvic ganglia (de Groat 2001). Parasympathetic afferents travelling in the hypogastric nerve innervate sympathetic fibers originating in the thoracolumbar spinal cord which stimulate contraction of the internal urethral sphincter and provide inhibition to the pelvic ganglia, thus suppressing parasympathetic efferent input to the detrusor smooth muscle (de Groat and Lalley 1972; de Groat and Theobald 1976). The inhibitory actions of parasympathetic afferents are removed when firing reaches a threshold analogous to a full bladder.

Voiding phase

The voiding phase of micturition involves a combination of spinal and spinobulbospinal reflexes incorporating parasympathetic, sympathetic and somatic pathways. The initiation of the voiding phase of micturition is turned on by a threshold level of bladder afferent firing and is mediated by activation of the sacral parasympathetic efferent axons to the bladder and urethra in combination with inhibition of sympathetic and somatic pathways to the urethral sphincters (de Groat 2006). The voiding phase, in contrast to the storage phase, is under voluntary control in adults through command of the EUS.

The bladder afferents travelling via the pelvic nerve synapse in the spinal cord and the post-synaptic neurons travel rostrally to the pontine micturition centre (PMC) in the brainstem (Blok, Willemsen et al. 1997). It has been shown that spinal cord injury above the lumbosacral level eliminates voluntary control of voiding and results in bladder hyperactivity (Yokoyama, Yoshiyama et al. 2000) and thus it is thought that the PMC co-ordinates the afferent sensory information with connections from the frontal cortex as well as other forebrain areas (Roberts 2008) to provide voluntary control of micturition.

Activation of spinobulbospinal pathways passing through the PMC stimulates parasympathetic pelvic efferents resulting in contraction of the bladder and relaxation of the urethral smooth muscle in combination with an inhibition of sympathetic efferent outputs (Drake, Fowler et al. 2010), and an inhibition of sphincter activity to give a flow of urine.

1.6 PATHOLOGIES OF THE BLADDER

The banner of urinary incontinence or lower urinary tract dysfunction encompasses a range of bladder pathologies. These include but are not limited to stress incontinence, urge incontinence, overflow incontinence and bladder outlet obstruction, and interstitial cystitis. These pathologies can occur individually or in combination whilst also varying extensively in their severity. Urinary incontinence has a huge impact on patients' quality of life and because it is so common, affecting approximately 4 million Australians according to the Australian Institute of Health and Welfare, it carries an enormous societal and economic burden estimated at AUD $1.5 billion annually.

The prevalence of bladder dysfunction is not evenly distributed across the population and is associated with a number of risk factors including age, pregnancy, and increased body mass index (BMI) (Hunskaar, Burgio et al. 2003; Rortveit, Daltveit et al. 2003). Age however is a major contributing factor, and, with an aging population the necessity to develop effective treatments, and improve quality of life is ever more present.

Overacitve Bladder

Overactive bladder (OAB) is defined by the International Continence Society (ICS) committee as 'urgency, with or without urge incontinence (approximately 30% of patients have incontinence Milsom et al (2001)), usually with frequency (voids ≥ 8/day (Wein and Rovner 2002)) and nocturia (Abrams, Cardozo et al. 2002) which occurs in the absence of infection or any other obvious pathology'. This complex of symptoms is believed to affect approximately 16-17% of the adult (≥40 years of age) population (Milsom, Abrams et al. 2001; Stewart, Van Rooyen et al. 2003). There is some overlap between the symptoms of OAB and interstitial cystitis (IC) except pain is not a defining feature of OAB, and therefore points towards a different aetiology.

As described earlier, during the filling phase of micturition the detrusor smooth muscle relaxes, allowing the bladder to fill with urine. OAB is associated with involuntary detrusor overactivity which is a urodynamic observation characterised by involuntary detrusor contractions during the filling phase, which may be spontaneous or provoked (Abrams, Cardozo et al. 2002). The detrusor, instead of exhibiting progressively increasing phasic activity correlating with an increased volume of urine and a progressive awareness of bladder distension up to the threshold for voiding, exhibits spontaneous contractile activity which causes an immediate rise in intravesical pressure and a desire

or ‘urgency’ to void, increased frequency of urination, and often urinary leakage. Overactive bladder syndrome can be separated into two main classes dependent on its aetiology:

- **Idiopathic overactive bladder**: has no definable cause and is not thought to be mediated by an inflammatory response such as that in interstitial cystitis. Most cases of OAB fall into this group.

- **Neurogenic overactivity bladder**: has an obvious neurogenic origin resulting in neurological dysfunction or degeneration of the neural pathways controlling micturition such as stroke, Parkinsons disease, multiple sclerosis, spina bifida, and spinal lesions, or any other disease associated with the nervous system.

The aetiology of detrusor overactivity is still unknown but is often considered to be multifactoral. A major concept now under intense investigation, is that of abnormal afferent firing from the bladder wall during the filling phase of micturition. This has been considered in respect to increased spontaneous activity of the detrusor myocyte (Andersson 2010), upregulation of C-fiber afferent activity and subsequent plasticity (Ouslander 2004), and increased activation of sensory C-fiber afferents in the sub-urothelium.

Stress urinary incontinence

Stress urinary incontinence (SUI) is defined by the ICS as ‘the involuntary leakage of urine during increased abdominal pressure, in the absence of detrusor contraction’ (Abrams, Cardozo et al. 2002) and is associated with involuntary leakage on everyday effort or exertion, for example sneezing, laughing or coughing. SUI is often attributable to a weakening of the urethral sphincters which allows urine to escape during periods of increased abdominal pressure. However, peripheral nerve dysfunction following damage to the pudendal nerve and weakening of the pelvic floor support during childbirth have also been described (Torrisi, Sampugnaro et al. 2007). Thus, pregnancy is a major risk factor for SUI and as a result is much more common amongst the female population (Luber 2004). SUI also increases definitively with age and although the pathophysiology governing this change is less well understood, it is believed to be due to the loss of urethral smooth muscle tone. Exercises to strengthen the pelvic floor muscles provide relief for a limited number of patients and there is also the option of surgery, but this is associated with increased risk factors and is not a viable option for many patients.

Overflow incontinence/Bladder outlet obstruction

Overflow incontinence is often defined as the involuntary loss of urine associated with bladder overdistention in the absence of detrusor contractions. This usually occurs as a result of inefficient voiding of the bladder which leads to excessive bladder pressure and the potential for urinary leakage. This form of incontinence is much more common amongst males and most commonly caused by prostate hyperplasia or prolapse of the pelvic organs which, through pressure exerted on the urethra, prevents the free flow of urine from the bladder. The current options for relief from overflow incontinence are limited and catheterisation remains the predominant method for alleviating the symptoms but does not address the root cause.

Painful Bladder Syndrome/Interstitial cystitis

Interstitial cystitis (IC) is a clinical diagnosis primarily based on symptoms of urgency/frequency and especially pain in the bladder. The ICS prefers the term painful bladder syndrome (PBS) and this is defined as 'the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and night-time frequency, in the absence of proven urinary infection or other obvious pathology' (Abrams, Cardozo et al. 2002). As with most bladder disorders the number of people suffering from IC is unclear, but is estimated at approximately 0.5 – 1% of the population (Jones and Nyberg 1997). A similar disorder occurs in domestic cats and is known as feline interstitial cystitis (FIC).

An alteration in the barrier function of the urothelium has been linked to the origin of IC. Histological analysis of the bladder has shown abnormalities in IC patients (Parsons 2007) which could underlie a change in the permeability of the urothelium and the increased access of noxious stimuli to the underlying chemosensitive neural network. This is exemplified by the increased pain observed with intravesical infusion of potassium in IC patients compared to healthy individuals (Parsons, Greene et al. 2005). The expression of inducible nitric oxide synthase was found to be increased in the FIC bladder, and increased levels of nitric oxide in mucosal studies have also been linked to altered barrier function (Birder, Wolf-Johnston et al. 2005). It could also be hypothesised that damage to the urothelium in IC results in an increased release of non-neuronal ATP from the urothelium, which can activate sensory afferents that would normally be quiescent. Urine samples from patients with IC have higher concentrations of ATP compared to controls, and greater stretch-induced ATP release from the urothelium (Sun, Keay et al. 2001). Furthermore, upregulation of $P2X_3$ receptors (Sun and

Chai 2004; Tempest, Dixon et al. 2004) has been described in IC patients and alterations of purinergic receptor expression in FIC cats (Birder, Ruan et al. 2004).

Neural plasticity of sub-urothelial nerve fibers is also observed in IC. An increased density of peptidergic afferent fibers innervating the bladders of cats and humans with IC has been reported, suggesting that abnormal afferent pathways could contribute to this disorder (Pang, Marchand et al. 1995; Buffington and Wolfe Jr 1998). This is also correlated to a significant increase in TRPV1 receptor expression on nerve fibers in IC patients (Mukerji, Yiangou et al. 2006) and this has been found to associate to pain scores. This observations are consistent with electrophysiological studies in which DRG neurons from FIC cats have abnormal excitability (Sculptoreanu, de Groat et al. 2005).

There is evidence, therefore, that IC and the pain integral to it, is a result of increased mediator release from the urothelium affecting afferent nerve activity. However, there also appears to be a significant inflammatory component in the maintenance of this pathology which is not evident in the other dysfunctions described here.

There is a growing consensus that release of a number of chemical mediators, in particular ATP from non neuronal sources such as the urothelium and acting on the afferent nerves innervating both the urothelium and sub-urothelium has a major part to play and that dysfunction or damage of the urothelium is a potential causality.

1.7 PURINERGIC RECEPTORS AND ATP

ATP is a universal energy source within cells. In the early 1970's it was proposed as the neurotransmitter responsible for the non-adrenergic, non-cholinergic component of neurotransmission (Burnstock, Campbell et al. 1970). The extracellular actions of ATP are mediated via a class of membrane bound receptors known as the P2 purinergic receptors (P indicating their activation by purines and in some cases pyramidine and the 2 discriminates them from the P1 receptors which are activated by adenosine). Following identification of ATP as a neurotransmitter, two types of P2 receptors were distinguished, P2X and P2Y, based on transduction mechanism studies, (Ionotropic P2X receptors and metabotropic P2Y receptors) (Burnstock 2009).

Purinergic receptors

P2X

There are seven ionotropic P2X receptors, $P2X_{1-7}$. All seven P2X receptors have intracellular N- and C-termini with protein kinase binding motifs, two transmembrane spanning regions separated by an extracellular domain, and an ATP binding site. P2X receptors show 30-50% sequence homology in amino acid sequence and are formed by the assembly of multiple subunits (North 2002) giving rise to distinct homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$ and heteromeric ($P2X_{2/3}$, $P2X_{1/5}$, $P2X_{4/6}$) channels.

Upon activation, P2X receptors are permeable to monovalent cations as well as a relatively high permeability to calcium. Homomeric P2X channels are distinguishable by their unique activation kinetics and their selective responses to a variety of agonist and antagonists. Heteromeric P2X channels compose agonist response phenotypes from the various properties of the constituent subunits. The endogenous ligand for all P2X receptors is ATP.

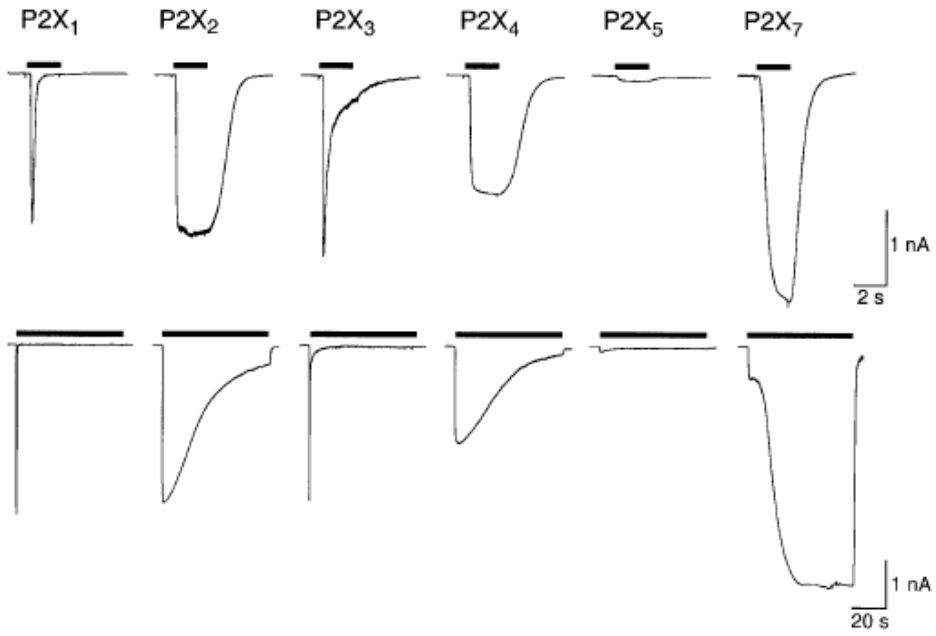

Fig 1.7.1 *Fast (top) and slow (bottom) desensitisation of rat homomeric P2X receptors* with ATP (30μM), except for $P2X_7$ (1mM) in HEK293 cells*. Significant responses and desensitisation are observed with all P2X receptor subtypes excluding $P2X_7$. Figure taken from North (2002).*

P2Y

The P2Y-receptor family consists of 8 human subtypes that have been cloned and functionally characterised. All eight P2Y receptors are typical G-protein coupled receptors comprising seven hydrophobic transmembrane domains connected by three intracellular and three extracellular loops, an extracellular N-terminus and intracellular C-terminus. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors all couple to stimulation of phospholipase C and the liberation of intracellular calcium via inistol trisphosphate (IP_3). In contrast, activation of the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors inhibits adenylate cyclase activity (von Kügelgen 2006). P2Y receptors are activated by the endogenous ligands ATP, ADP, UTP and UDP. P2Y receptors can generally be characterised by their rank order of potency for particular agonists as shown in table 1.7.1.

Ectonucleotides

Purinergic signalling is not only regulated via activation of purinergic receptors, but also through the actions of ectonucleotides which rapidly degrade ATP and UTP to their relevant nucleotides. Ectonucleotides act to reduce the concentration of ATP and UTP at the receptor, thus preventing

overstimulation and desensitisation of receptors (particularly P2X), but through the process of breakdown into intermediates such as ADP and UDP, produces a dynamic signalling pathway allowing for the activation of diverse subtypes of purinergic receptor. Ectonucleotide breakdown is eventually responsible for the termination of the purinergic response.

Receptor	Endogenous agonist
$P2X_{1-7}$	ATP
$P2Y_1$	ADP > ATP
$P2Y_2$	UTP = ATP
$P2Y_4$	UTP > ATP
$P2Y_6$	UDP >> UTP > ATP
$P2Y_{11}$	ATP > UTP
$P2Y_{12}$	ADP >> ATP
$P2Y_{13}$	ADP >> ATP
$P2Y_{14}$	UDP-glucose

Table 1.7.1 *Rank order of potency of P2X and P2Y receptors for endogenous agonists. Adapted from Alexander (2011)*

Within the bladder it has been found that ectonucleotidases are expressed throughout the bladder. Specifically, E-NTPDase enzymes for the metabolism of ATP to ADP and AMP were found in the suburothelium and on the basal and intermediate cells of the mouse urothelium (Yu, Robson et al. 2011). This coupled with the presence of multiple purinergic receptors on the urothelium and underlying interstitial cells contributes to the hypothesis that ATP breakdown is not solely for the aim of preventing desensitisation, but is an essential component in the synchronisation of purinergic signalling. This has become increasingly apparent with recent findings that adenosine exerts a powerful negative feedback control of ATP release from the urothelium via P1 receptors (Dunning-Davies, Fry et al. 2013).

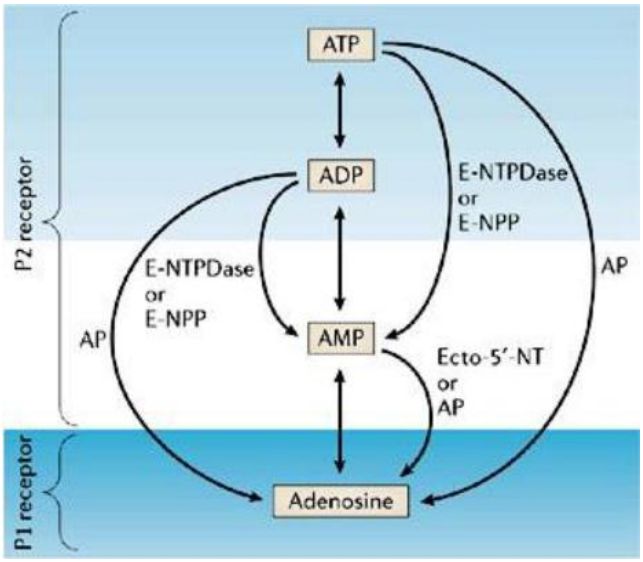

Fig 1.7.2 *The action of several ectonucleotidases on the metabolism of ATP into ADP, AMP and Adenosine. Abbreviations: ectonucleoside triphosphate diphosphohydrolase, (E-NTPDase); ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP); (Ecto-5'-NT) Ecto-5'-nucleotidase; (AP). Taken from (Fields and Burnstock 2006).*

ATP in the bladder

A more comprehensive review of the actions of ATP will be covered in the relevant results chapters, however, whilst running the risk of repetition, a more concise description will be given here.

ATP has been shown to be co-released with acetycholine from parasympathetic nerve terminals innervating the bladder (Burnstock 2009) and acts on purinergic receptors to initiate contraction of detrusor smooth muscle. The relative contribution of ATP to nerve-mediated contraction is species dependent and in the healthy human bladder it appears to be zero (Andersson and Wein 2004). However, an important observation is that in detrusor strips from both male and female patients with a range of lower urinary tract disorders, a significant purinergic component emerges (Ford, Gever et al. 2006).

The main focus of this thesis however, is the role of non-neuronal ATP released from the urothelium, and this will be discussed further.

ATP release

The synthesis and storage of ATP within cells is essential to its autocrine and paracrine actions within the bladder. ATP is stored at high concentrations, reported to be within the range of 5mM, in

secretory vesicles of neurons. It could be assumed that due to the roles of ATP within the bladder, these concentrations would be of an equivalent magnitude. Bladder distension, or stretch of the urothelial cell layer has been shown to induce ATP release (Ferguson, Kennedy et al. 1997; Sadananda, Shang et al. 2009; Collins, Daly et al. 2013) through an intracellular calcium dependent (Matsumoto-Miyai, Kagase et al. 2011; Dunning-Davies, Fry et al. 2013), vesicular exocytosis mechanism (Knight, Bodin et al. 2002; Birder, Barrick et al. 2003) which is inhibited by botox (Collins, Daly et al. 2013). Distension induced urothelial ATP release has been shown to increase in age (Yoshida, Homma et al. 2001), models of spinal cord injury, cyclophosphamide induced inflammation, and feline interstitial cystitis (Sun and Chai 2002; Khera, Somogyi et al. 2004; Smith, Vemulakonda et al. 2005; Smith, Gangitano et al. 2008). ATP is also increased in human luminal samples from patients with OAB and ATP has consequently been proposed as a biomarker for this disorder (Cheng, Mansfield et al. 2013; Silva-Ramos, Silva et al. 2013). Thus a role for non-neuronal ATP in modulating sub-urothelial structures, particularly the afferent nerves in control of mediating the micturition reflex has been proposed.

ATP and mechanotransduction

As mentioned earlier in this introduction, an indirect pathway for the transduction of mechanical stimuli from the bladder has been proposed and non-neuronal ATP is central to this hypothesis.

The concept of ATP as a sensory neurotransmitter was first discussed by Holton et al in 1959 but was not accepted as such for a great number of years (Holton 1959). The work of Burnstock et al was significant in concluding that ATP in fact was the NANC neurotransmitter release from inhibitory nerves in the gut (Burnstock, Campbell et al. 1970). The release of ATP from the urothelium of the rabbit in response to hydrostatic pressure was the first to show that ATP was released from non-neuronal structures and it was postulated that it could stimulate the plexus of nerves lying beneath the urothelium (Ferguson, Kennedy et al. 1997), and thus act as a sensory neurotransmitter. This work was the basis of the 'purinergic hypothesis' put forward by (Burnstock 1999) that distension induced ATP release from the urothelium acts on the $P2X_2$ or $P2X_3$ receptors of the sub-urothelial sensory afferents to convey information to the CNS regarding the filling state of the bladder. This hypothesis is supported by the release of ATP from the urothelium in response to stretch (Sadananda, Shang et al. 2009; Collins, Daly et al. 2013), and immunohistochemical localisation of $P2X_3$ receptors on the sub-urothelial afferent nerves (Vlaskovska, Kasakov et al. 2001; Brady, Apostolidis et al. 2004; Apostolidis, Popat et al. 2005).

A number of functional studies have linked ATP and P2X receptors to bladder mechanosensation, and the use of multiunit afferent recording techniques have provided further insight. Briefly, it has been observed that pharmacological blockade of $P2X_3$ receptors significantly reduced sensory nerve firing (Namasivayam, Eardley et al. 1999) and that knock-out of the $P2X_3$ and $P2X_{2/3}$ receptors significantly attenuate the afferent discharge in response to ramp distension (Vlaskovska, Kasakov et al. 2001; Cockayne, Dunn et al. 2005). Importantly, an endogenous role for urothelially released ATP has been shown by a decrease in the afferent nerve response with administration of a P2X receptor antagonist (Rong, Spyer et al. 2002).

1.8 TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1)

The transient receptor potential (TRP) channel family are comprised of 7 structurally related proteins, TRPA, TRPC, TRPM, TRPML, TRPP, TRPN and TRPV. The TRPV subfamily currently contains 6 members, TRPV1-6 (Clapham 2003). All TRP channels are characterised by six-transmembrane polypeptide subunits that assemble to form a pore region between transmembrane domains (TM) V-VI (Minke and Cook 2002) as well as intracellular amino (N-) and carboxyl (C-) terminal cytosolic domains. The N-terminus contains at least three ankyrin repeats which are essential to channel function (Jung, Lee et al. 2002; Tominaga and Tominaga 2005).

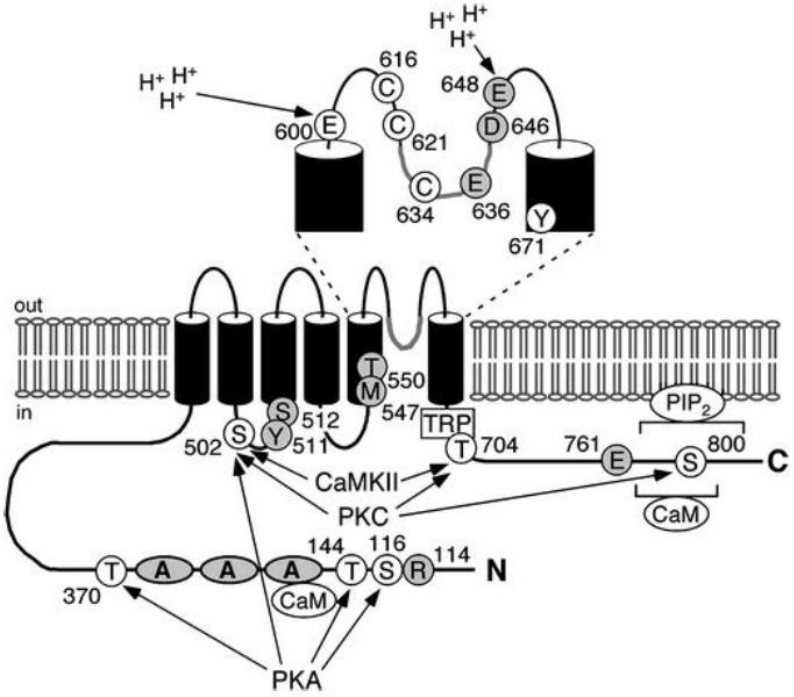

Fig 1.8.1 *Extracellular activation and intracellular amino acids involved in TRPV1 function, residues believed to be involved in vanilloids binding are in grey. TRP; TRP domain. Phosphatidylinositol (4,5)-bisphosphate (PiP_2) binds to region indicated. Calmodulin (CaM) binds to both C and N termini. A indicates the ankyrin domain. Protons act at GLU600 in the extracellular loop as indicated by arrows. Protein kinase A (PKA), protein kinase C (PKC), or calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylate Ser (S) or Thr (T) residues as indicated by arrows. Fig taken from Tominaga & Tominaga (2005).*

Activation and desensitisation of TRPV1.

TRPV1 is activated by vanilloid compounds (such as capsaicin and resiniferatoxin), noxious temperature (> 44˚C), and acid (<pH 6.0) and thus has an essential role in the amalgamation of noxious stimuli. Endogenous ligands for TRPV1 include the endocannabinoid anandamide (Zygmunt, Petersson et al. 1999), and other derivatives of long-chain fatty acids (Ahern 2003; Matta, Miyares et al. 2007). Protons have been shown to sensitise the TRPV1 receptor to temperature activation to the extent that even moderately acidic conditions (pH ≤ 5.9) activate TRPV1 receptors at room temperature (Tominaga, Caterina et al. 1998; Jordt, Tominaga et al. 2000). The threshold for TRPV1 activation is reduced by inflammatory mediators such as NGF (Chuang, Prescott et al. 2001), bradykinin (Chuang, Prescott et al. 2001), and ATP (Tominaga, Wada et al. 2001) through the hydrolysis of the phospholipid PiP_2.

Upon activation, the TRPV1 receptor is permeable to both monovalent and divalent cations but exhibits higher specificity for divalent ions, and particularly high calcium permeability resulting in voltage dependent depolarisation. The calcium component of TRPV1 receptor activation is especially important as extracellular calcium is essential for receptor desensitisation (Koplas, Rosenberg et al. 1997), and thus the dynamic adaption of TRPV1 expression in sensory afferents to continuous or repeated stimuli (Rosenbaum, Gordon-Shaag et al. 2004), which is also the proposed mechanism involved in TRPV1 mediated analgesia.

Desensitisation of the TRPV1 receptor is not fully understood but is believed to occur in two stages: acute desensitisation, followed by permanent disruption of channel function (Koplas, Rosenberg et al. 1997). As mentioned above, an increase in intracellular calcium is essential for TRPV1 desensitisation, and calmodulin has been suggested to have an important role in this mechanism (Rosenbaum, Gordon-Shaag et al. 2004) as well as channel dephosphorylation by calcineurin (Mohapatra and Nau 2005). cAMP-dependent protein kinase A has been shown to decrease desensitisation by directly phosphorylating TRPV1 (Bhave, Zhu et al. 2002) and replenishment of PiP_2 in the membrane determines recovery of the channel from desensitization (Liu, Zhang et al. 2005). These processes are thought to mediate the short term desensitisation of TRPV1, but the long term receptor down-regulation that occurs within intravesical administration of capsaicin in the bladder is, however, less well understood. A current theory focuses on TRPV1 withdrawal from the cell surface, and recent evidence has shown that long-term neuronal disruption occurs through clathrin-independent internalisation and endocytosis (Sanz-Salvador, Andrés-Borderia et al. 2012).

Where are TRPV1 channels found?

The TRPV1 receptor is found in a number of non-neuronal cells, though its major expression is upon peptide-containing thinly myelinated Aδ and unmyelinated C-fibers including DRG neurons. Whilst also expressed in the spinal cord and brain of the central nervous system (Winter 2005). Peripheral sensory neurons express TRPV1, with most TRPV1 immunolabelling occuring within capsaicin sensitive C-fibers but overlaps with CGRP immunoreactivity or isolectin B4 (IB4). There is also limited expression of TRPV1 in Aδ-fibers of DRG neurons. In the spinal cord, TRPV1 is predominantly expressed on sensory afferent terminals, consistent with C- and Aδ-fiber expression (Guo, Vulchanova et al. 1999; Guo, Simone et al. 2001) as well as in trigeminal ganglia (Ichikawa and Sugimoto 2001). Interestingly, Meissners corpuscles, responsible for a portion of somatic mechanosensation and found within the skin are innervated by TRPV1 immunofluorescent fibers (Paré, Elde et al. 2001) suggesting a mechanosensitising role.

Of major interest to this project is the presence of TRPV1 within the bladder, particularly pertaining to those receptors which are likely to influence afferent nerve excitability and discharge. Many immunohistochemical studies have demonstrated the presence of TRPV1 in the dorsal root ganglia neurons (Hwang, Min Oh et al. 2005) supplying the bladder and on nerve fibers throughout the lower urinary tract. There is significant immunoreactivity for the TRPV1 receptor within the bladder, the majority of which is reported on non-myelinated C-fibers (Tominaga, Caterina et al. 1998; Avelino, Cruz et al. 2002), but also larger diameter A-fibers (Yiangou, Facer et al. 2001; Ost, Roskams et al. 2002) present in muscular and suburothelial plexuses. As well as the documented presence of TRPV1 within the peripheral endings of sensory afferents there is evidence of TRPV1 immunoreactivity within interstitial cells (Ost, Roskams et al. 2002), smooth muscle cells (Birder, Kanai et al. 2001; Ost, Roskams et al. 2002), and more controversially, the urothelium, where TRPV1 expression is seen by some (Birder, Kanai et al. 2001; Lazzeri, Vannucchi et al. 2004; Apostolidis, Brady et al. 2005) and not by others (Ost, Roskams et al. 2002; Everaerts, Sepúlveda et al. 2009; Yamada, Ugawa et al. 2009) with no discernible explanation for the discrepancies apart from different immunohistochemical techniques and the use of a number of different species.

The role of TRPV1 in the bladder

Pathophysiology

A number of studies report a role for TRPV1 in bladder pathophysiology, inflammation, and pain, which are thought to be mediated by capsaicin sensitive primary afferent neurons (CSPANS). Patients with neurogenic detrusor overactivity show a significant increase in neuronal and urothelial TRPV1 immunoreactivity within the bladder (Brady, Apostolidis et al. 2004; Apostolidis, Brady et al. 2005), and intravesical vanilloid treatment (resiniferatoxin/capsaicin) has been successfully used for the treatment of lower unirary tract symptoms associated with sensory urgency and pain including idiopathic and neurogenic detrusor overactivity, and interstitial cystitis (De Ridder, Chandiramani et al. 1997; Chancellor and de Groat 1999; Silva, Silva et al. 2007). In addition, intravesical application of resiniferatoxin in patients with neurogenic detrusor overactivity caused a reduction in immunoreactivity for TRPV1 (Brady, Apostolidis et al. 2004; Apostolidis, Brady et al. 2005), supporting the idea that the TRPV1 receptor is essential for bladder sensation, particularly in disease states. Likewise, in various murine models of inflammation, including cyclophosphamide, acrolein, and lippopolysaccharide, the TRPV1 receptor has been shown to be essential in the development of mechanical hyperactivity in the bladder, by using either pharmacological blockade (Vizzard 2000; Dinis, Charrua et al. 2004), or knockout of the TRPV1 receptor (Charrua, Cruz et al. 2007; Wang, Wang et al. 2008). The mechanisms determining this alteration in sensory function, however, are still in question.

Physiology

There is ubiquitous expression of the TRPV1 receptor within the bladder, yet the role of TRPV1 in normal bladder function lacks the conclusive role observed in animal and human models of inflammation. A role for the TRPV1 receptor in normal bladder mechanosensation has become apparent with the use of TRPV1 knockout ($TRPV1^{-/-}$) mice. Birder et al (2002) showed that $TRPV1^{-/-}$ (KO) mice exhibit reduced reflex voiding and spinal cord signalling and it has since been shown that $TRPV1^{-/-}$ (KO) mice demonstrate reduced multiunit afferent responses to bladder distension (Daly, Rong et al. 2007). $TRPV1^{-/-}$ (KO) mice exhibit blunted responses to jejunal and colorectal distension in vitro (Rong, Hillsley et al. 2004; Jones, Xu et al. 2005) which is also attenuated with pharmacological blockade of TRPV1.

In the bladder, the TRPV1 receptor has been shown to mediate distension induced ATP release from the urothelium (Birder, Nakamura et al. 2002; Sadananda, Shang et al. 2009; Dunning-Davies, Fry et al. 2013), and thus a role for TRPV1 in mechanosensation mediated via an indirect pathway has been proposed.

1.9 TACHYKININS

Tachykinin receptors

The tachykinins are a family of peptides consisting primarily of substance P, Neurokinin A (NKA), and Neurokinin B (NKB). It has been well established that tachykinins are present within the central and peripheral nervous system. Substance P and NKA are found in the peripheral nervous system, confined primarily to primary afferent sensory neurons innervating a number of peripheral structures, including the urinary bladder. NKB is mostly found within the spinal cord and central nervous system (Mar, Yang et al. 2012). The tachykinin neuropeptides are encoded by the genes TAC 1 (PPT-A) for substance P and NKA, and TAC 2 (PPT-B) for NKB. Tachykinins are synthesised in the endoplasmic reticulum before passing via the Golgi to form the active peptide molecule and packaged into secretory vesicles then transported to the nerve terminals (Pennefather, Lecci et al. 2004). Tachykinins were considered to function entirely as neuropeptides until relatively recently when they were identified outside the nervous system (Severini, Improta et al. 2002).

There are three major classes of tachykinin receptors, known as NK1, NK2 and NK3. Tachykinin receptors belong to a family of G-protein coupled receptors consisting of 7 hydrophobic transmembrane domains (TM I-VII) with three extracellular and intracellular loops, an extracellular amino terminus and a cytoplasmic carboxyl terminus (Maggi 1995). Endogenous tachykinins bind to tachykinin receptors with only moderate selectivity. All three tachykinins, substance P, NKA and NKB bind to, and act as full agonists for all three tachykinin receptor subtypes but show a defined order of potency such that substance P binds preferentially to NK1, NKA preferentially binds NK2, and NKB is more selective for NK3 (Mussap, Geraghty et al. 1993; Regoli, Boudon et al. 1994; Maggi and Schwartz 1997).

There is evidence of additional tachykinin receptors and the existence of tachykinin isoforms but because there are few pharmacological tools to distinguish them they will not be considered further in this thesis.

Why the interest in Tachykinins?

As mentioned previously, intravesical instillation of vanilloids (capsaicin and resiniferatoxin) show positive outcomes when used in the treatment of both idiopathic and neurogenic bladder disorders

yet the precise physiology behind their function is far from understood. Capsaicin, and related compounds bind to the TRPV1 receptor, expression of which is mostly confined to unmyelinated, small diameter primary afferent fibers known to be capsaicin sensitive (CSPANS). High doses of capsaicin have the ability to first activate, desensitise and eventually permanently disrupt sensory function. This neurotoxic effect of capsaicin on primary afferent neurons is accompanied by a significant reduction in tachykinin immunoreactivity (Maggi 1993; Avelino and Cruz 2000; Lecci and Maggi 2001). Stimulation of CSPANS with a non-desensitising dose of capsaicin is known to trigger the release of tachykinins and the resultant contractile response of the detrusor smooth muscle can be blocked by prior administration of tachykinin receptor antagonists (Lecci, Giuliani et al. 1997).

In general, intravesical instillation of vanilloids like capsaicin and resiniferatoxin produces positive effects on bladder symptoms by increasing bladder capacity and decreasing sensations of urgency and frequency (Lazzeri, Spinelli et al. 1998; Silva, Rio et al. 2000). It has been proposed that these beneficial effects are mediated by changes in the release of endogenous tachykinins. However, the precise roles that tachykinin receptors play within the bladder, and particularly their role in sensory signalling are yet to be determined.

Where do tachykinins come from?

Tachykinin neuropeptides are primarily contained within capsaicin sensitive primary afferent neurons (Lecci and Maggi 2001; Avelino, Cruz et al. 2002) but there is also building evidence that tachykinins are present in capsaicin-insensitive neurons, primarily following inflammation or insult (Neumann, Doubell et al. 1996; Hunter, Myers et al. 2000; Carr, Hunter et al. 2002). Tachykinins are also present in the enteric nervous system within the gastrointestinal tract (Lomax and Furness 2000). Tachykinins have been identified in a range of non-neuronal tissue including, but not limited to epithelial cells (Chu, Kraft et al. 2000), and immune cells, in which substance P/NKA act as mediators of neurogenic inflammation and neuroimmunomodulation (Maggi 1997; Pennefather, Lecci et al. 2004). Maggi et al (1988) proposed a mechanism in which CSPANS have a dual role in sensory signalling: normal afferent sensory signalling from the nerves to the spinal cord is mediated in part by CSPANS, but the sensory stimulus also triggers the peripheral terminals of certain sensory neurons to release stored neuropeptides to evoke an effector response as part of an axon reflex, which has been termed a sensory ‘efferent’ function. This is supported by more recent experiments which have shown that CSPANS synthesise and release substance P and NKA (Hökfelt, Pernow et al. 2001) and also express the receptors for these neuropeptides (Brechenmacher, Larmet et al. 1998).

CSPANS also exhibit enhanced excitatory responses to external stimuli following NK2 receptor activation (Sculptoreanu and de Groat 2007; Sculptoreanu, Artim et al. 2009). It is therefore proposed that neurokinins may act as an autofeedback modulator of afferent nerve sensitivity.

Expression in the bladder

In most species, nerves immunoreactive for NKA are found in the detrusor muscle (Burcher, E et al. 2000; Andersson 2002) but more densely populate the sub-urothelium, encircling intramural ganglia, where they are typically co-localised with substance P and CGRP (Smet, Edyvane et al. 1996; Smet, P et al. 1997). It has also been suggested that tachykinin-immunoreactive fibers are more abundant in the bladder base than in the dome (Maggi 1993). Furthermore, NK2 receptor mRNA has recently been observed in cultured suburothelial cells (Bahadory, Moore et al. 2013).

Changes in expression

In the sub-urothelium of patients with idiopathic detrusor overactivity there is a significant increase in tachykinin immunoreactive fibers (Moore K H 1992; Smet, Moore et al. 1997) and it is proposed that alterations in micturition are the result of afferent nerve activation via tachykinin receptors in the sub-urothelium either directly or via smooth muscle contraction. Callsen-Cencic et al (1997) also confirmed bladder projecting DRG neurons which innervate the rat bladder contain tachykinins and showed that cystitis induced hyperreflexia caused a significant increase in the number of DRGs expressing tachykinins. However, due to the lack of specific action of substance P in the periphery, it was proposed that the increase in substance P-immunoreactive bladder afferents in lumbosacral regions enhanced activation of the excitatory parasympathetic pathway to the urinary bladder, and contributed to the hyper excitability of micturition reflexes in cystitis rats (Callsen-Cencic and Mense 1997). This provides a mechanism where an ‘efferent’ function of neurokinins released from the peripheral endings of these sensory afferent nerves can combine with central effects in the spinal cord to promote hyperreflexia.

Role in Inflammation and disease

Alterations in the expression of tachykinin containing afferent nerves and a role in bladder disease and inflammation have been thoroughly investigated. Increases in tachykinin immunoreactive afferent nerves in the bladder have been observed in human detrusor overactivity (Moore K H 1992; Smet, Moore et al. 1997), OAB (Tyagi, Tyagi et al. 2013), and interstitial cystitis patients (Liu, Yang et al. 2014). This is also true for animal models of chronic inflammation (Callsen-Cencic and Mense 1997), ischemia (Azadzoi, Radisavljevic et al. 2008) and diabetic cystopathy (Philyppov, Paduraru et al. 2012). Inflammatory markers such as nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) have also been implicated in mediating the changes in tachykinin expressing neurons (Saban, Davis et al. 2011; Girard, Tompkins et al. 2012), and consequent changes in bladder function (Schnegelsberg, Sun et al. 2010; Malykhina, Lei et al. 2012; Liu, Yang et al. 2014).

These results thus provide a link by which inflammation can induce recruitment of CSPANS and an increase in bladder content of tachykinins which is able to influence bladder function.

Contraction and micturition

Tachykinin neuropeptides have thus been implicated in pathophysiological bladder function, yet the site of action of these proposed modulators is still unclear and their role in normal bladder physiology has not been elucidated. There has been a distinct lack of experimental research concerning tachykinins in the bladder, however, a role for tachykinins in the regulation of detrusor smooth muscle tone, release of urothelial mediators, and as modulators of afferent sensitivity as briefly described above have all been proposed.

NKA, acting via the NK2 receptors is the sole tachykinin responsible for stimulating detrusor muscle contractions in a number of species (see (Candenas, Lecci et al. 2005) for review) but the NK1 receptor has also been shown to play a part role in both the rat and guinea pig detrusor contractions (Suman-Chauhan, Guard et al. 1990; Longmore and Hill 1992), where NK1 and NK2 receptor mediated responses summate. The urothelium has been shown to express mRNA for the NK2 receptor (Bahadory, Moore et al. 2013), and has also been implicated in NKA mediated effects within the bladder through the alteration of mediator release, chiefly nitric oxide and prostanoids (Tramontana, Catalioto et al. 2000; Candenas, Lecci et al. 2005). Intravesical instillation of NKA into the bladder lumen has also been shown to induce micturition, and it was proposed that these actions were secondary to effects mediated via the urothelium (Maggi, Giuliani et al. 1991; Ishizuka,

Mattiasson et al. 1995). There is also some evidence to suggest that NKA may also regulate interstitial cell excitability and mucosal contractions (Sadananda, Chess-Williams et al. 2008).

Conclusions

The body of work described above has provided evidence for the roles of ATP and purinergic receptors in mediating afferent nerve activity arising from the bladder. Specifically, a role for non-neuronal ATP released from the urothelium during distension is consistently implicated in the activation of indirect mechanosensitive pathways. The ability of this mechanism to influence the sensory structure which forms between the urothelium and the underlying sensory afferent nerves appears essential to maintaining urinary continence. The P2, TRPV1 and tachykinin receptors, as well as sensory neuropeptides expressed within the bladder, have also been shown to have essential roles in mediating bladder afferent activity through both the urothelium and sensory afferent nerves and this thesis will investigate the coordination of these pathways.

1.10 AIMS

The overall aim of this thesis was to gain new information on the mechanisms involved in afferent transduction and mechanosensitivity in the bladder, and the contribution of non-neuronal ATP to this process. Understanding the sensory structures which control afferent nerve excitability and the stimulus for afferent nerve firing provides essential information regarding the micturition cycle, and has the potential to reveal future targets for the pharmacological intervention of lower urinary tract disorders. This thesis draws on a number of different experimental approaches to address the following specific aims.

Specific Aims

- To elucidate the expression of purinergic receptors by the mouse urothelium, and determine their relative contribution to functional responses gauged from increases in intracellular calcium that represents an essential component of non-neuronal ATP release.

- Examine the possible functional interaction between TRPV1 and P2X mediated mechanosensitivity in bladder afferent nerves, detrusor smooth muscle, urothelial cells and isolated DRG neurons using pharmacological blockade and genetic deletion of the TRPV1 channel.

- Investigate the role of NK1, NK2 and NK3 receptors, and endogenously released tachykinin peptides in the control of bladder mechanosensitivity via actions on the detrusor smooth muscle, afferent nerves and urothelial cells. The role of the NK2 receptor as a modulator of P2X mediated mechanosensitivity was also investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMALS

All experiments were performed using adult male mice on a C57 black background (14-16wk, 25-30g). TRPV1 wildtype (WT) and knockout (KO) mice with a genetic background of C57/BL6 were generated by GlaxoSmithKline (Harlow, UK). Trans-membrane domains 2-4 of the mouse VR1 gene (i.e. DNA encoding amino acids 460-555) were replaced by the neo gene (Davis, Gray et al. 2000). Mating pairs of $TRPV1^{-/-}$ (KO) and $TRPV1^{+/+}$ (WT) N1F1 littermates were obtained to generate separate colonies of TRPV1 WT and KO mice at the University of Sheffield according to the UK Animals (Scientific Procedures) Act 1986. In the course of the study, genotyping was periodically performed to confirm the absence of the TRPV1 gene in KO mice. There were no overt differences in feeding behaviour, litter size, growth rate and body weight between WT and KO groups.

For experiments performed in Australia, adult male mice on a C57 black background were also used, and obtained through Griffith University animal house in accordance with ethics approved through Griffith University Ethics committee #MSC/04/11/AEC.

All animals were allowed free access to food and water and were humanely killed by cervical dislocation in accordance with UK home office regulations covering schedule one procedures whilst in the UK and in accordance with Australian ethical committee approval and the guidelines of the national health and medical research council of Australia whilst in Australia.

Tissues obtained from mice were used in all experiments and composed a number of separate protocols. These included: Recording intravesical pressure and afferent nerve activity, harvesting of urothelial cells and DRG neurons for calcium imaging, qPCR and immunohistochemistry, and the use of the whole bladder for mediator release studies.

2.2 IN-VITRO AFFERENT NERVE RECORDING

Following cervical dislocation of mice, an incision in the abdomen was made in order to remove the gastrointestinal tract visible superior to the bladder. The animal was cut in two at the level of L2, removing vascular connections via the aorta and vena cava, yet ensuring the kidneys and ureters remained intact. The tail and hind limbs were removed and the remaining pelvic section consisting of bladder, testes, urethra, ureter and kidneys was placed into ice cold Krebs solution whilst being transported to the recording chamber. The recording chamber was continually perfused with oxygenated (95% O2 and 5% CO_2) Krebs-bicarbonate solution (composition, mM: NaCl 118.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 11.7) at a stable temperature of 35°C to maintain functioning tissue.

Under a dissection microscope (WPI, PZMIII), the ureters were identified and ligated using silk suture (US 4/0) to prevent back flow. Excess tissue surrounding the bladder neck was removed to expose the pubic symphasis. The pubic symphasis was cut along the rostral/caudal axis of the urethra and connective tissue attaching them together was removed. The pelvic bone on either side of the urethra was cut away to the point of attachment to the hind legs to provide increased visibility and ease of dissection for the pelvic nerves. The urethral ending was cut and a catheter (OD 0.03IN) attached to a syringe pump (Genie, Kent, multi-phaser™ model NE-1000) was inserted into the bladder and tied in place using silk suture. The bladder was then filled with 0.9% saline to create a small amount of pressure in order to pierce the bladder dome with a syringe needle (BD microlance™, 19G 2”). A double lumen catheter, connected to both a pressure transducer (DTX™ plus DT-XX, Becton Dickinson, Singapore), to allow recordings of intravesical pressure, and an outflow (two-way) tap was then inserted into the bladder dome through the incision and secured in place with silk suture. The open or closed state of the tap allowed control over filling and emptying the bladder

Following catheterisation, bladder nerve bundles containing both pelvic and hypogastric afferent nerves exiting the bladder were located around the base of the bladder, proximal to the point at which it connects to the urethra. The bladder nerve bundles converge with urethral bundles as they run dorsally towards the spinal cord and it is here that they are most easily identified under the microscope. Gently removing connective and fatty tissue exposed the nerve bundles which were carefully divided into individual strands. A nerve strand was selected and inserted into a suction electrode (tip diameter 25-50μm) attached to a Neurolog headstage (NL100, digitimer, Ltd, UK)

which allowed the recording of multi-unit afferent nerve activity. The NL100 was connected to an AC pre-amp (NL014) allowing the amplification of the nerve signals (x10,000) before they were filtered (NL125, band pass filter) and passed through a 50/60Hz electrical noise eliminator (Humbug, Quest Scientific) and finally passed via a micro 1401 analogue to digital interface and visualised on a computer using Spike 2 software (version 7.1, Cambridge Electronic Design, UK).

Whole nerve multifiber afferent nerve activity was quantified using the spike 2 software which counted the number of action potentials crossing a pre-set threshold.

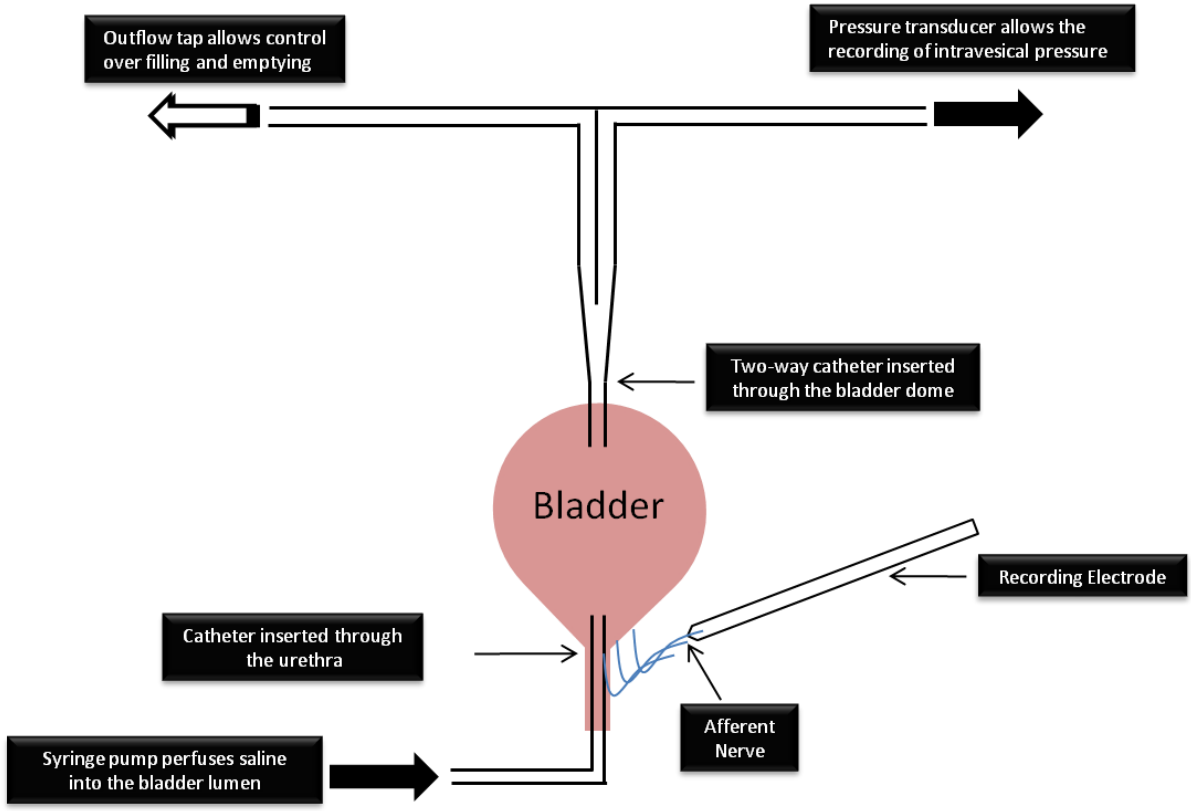

Fig 2.2.1 Schematic diagram representing an In vitro model for recording intravesical pressure and afferent nerve firing of the bladder. *The urethral catheter was attached to a syringe pump allowing infusion of saline/drugs into the lumen of the bladder. A double lumen catheter inserted into the bladder dome was attached to a pressure transducer to allow monitoring of intravesical pressure, as well as an outflow tap from which to empty the bladder. Bladder afferent nerve bundles were identified, and inserted into a recording electrode. Action potentials were filtered, amplified and recorded using computer software.*

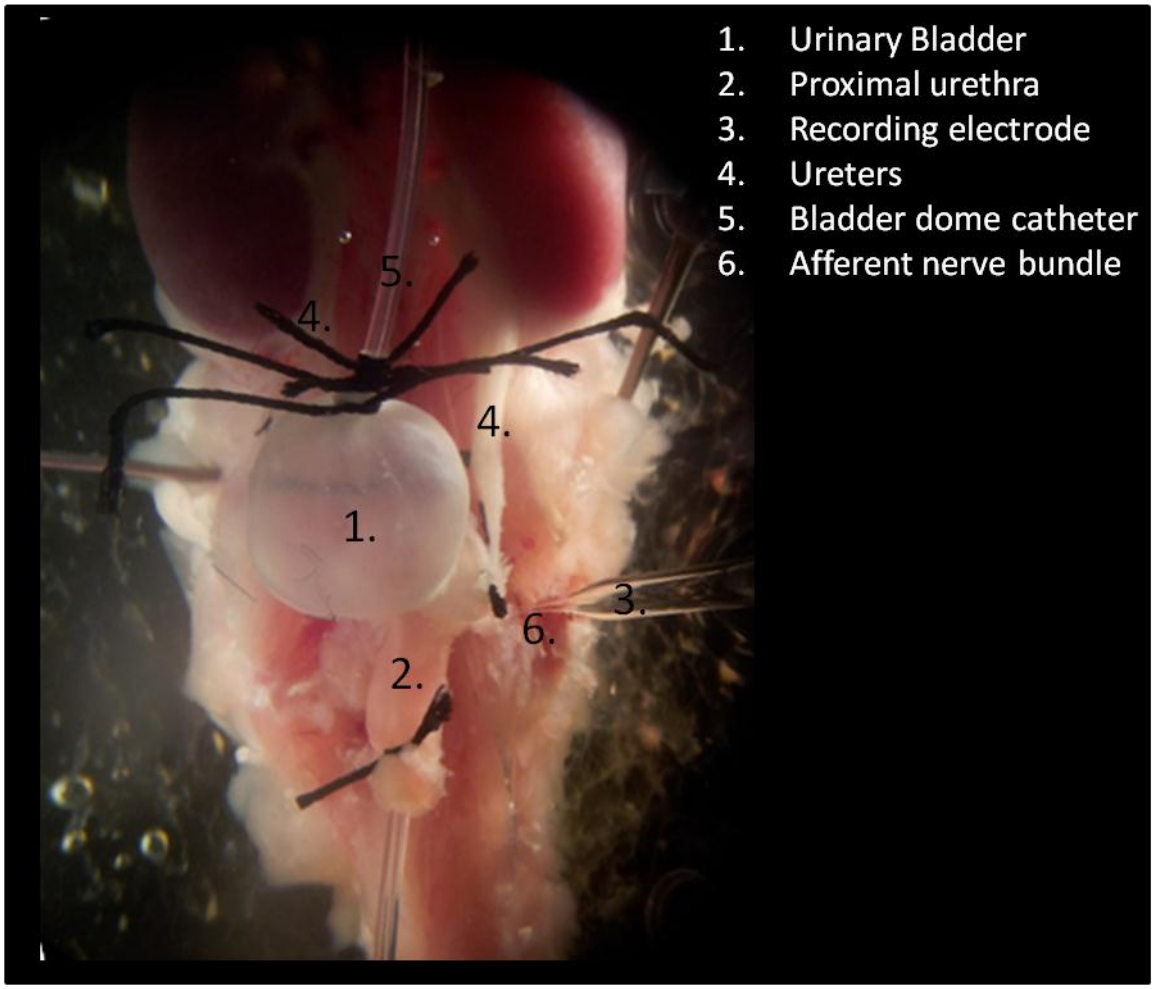

Fig 2.2.2 *Photograph of the in-vitro experimental set-up for recording intravesical pressure and afferent nerve firing of the bladder.*

2.3 DATA ANALYSIS

Distension

Multi unit nerve activity was quantified through Spike software which counted the number of spikes that cross a pre-set threshold; with the threshold level for spike counting set depending on the parameters to be measured. Further simultaneous analysis quantified the afferent nerve activity in a sequential rate histogram. Baseline afferent activity was obtained by averaging the discharge in the 100 second period prior to the distension. The afferent nerve response during bladder distension was calculated by measuring the afferent activity (action potentials/per second) at various intravesical pressures (0-40mmHg). These values were then substracted from the baseline firing to give a total change in afferent nerve response during distension.

Baseline firing

During application of drug without distension of the bladder, baseline firing was calculated by measuring the peak response after drug application and then subtracting the mean baseline firing (100 seconds) prior to application of the drug.

Bladder compliance

Bladder compliance is the ability of the bladder to accommodate the volume within, in relation to the intravesical pressure.

$$\textbf{Volume (µl) = Rate (µl/min}^{-1}\textbf{) x Time (s)}$$

The pressure/volume relationship (compliance) was calculated at various intravesical pressures (0-40 mmHg) during bladder filling and determined from the rate of infusion (µl/min^{-1}) and the time (s) from the start of the infusion. Compliance was then represented as a pressure-volume XY plot.

Afferent nerve activity

Afferent nerve activity is expressed as impulses per second (imp/s^{-1}). Data are expressed as means ± S.E.M. Statistical analysis was carried out using either a 2-way ANOVA followed by a Bonferroni multiple comparisons post test where necessary or one–way ANOVA/paired students t test and significance was set at $P< 0.05$. (All graphical and statistical analysis used in this thesis was

performed using Graph Pad Prism (Version 5.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com).

2.4 EXPERIMENTAL PROTOCOL

Afferent nerve recordings

The recording of multiunit nerve preparations was performed whilst the bladder was both at rest and under distension.

Preparation viability

Before the start of each experiment, the bladder was filled with saline to allow an examination of the viability of the experimental preparation. Bladders which did not fill to the set threshold volume of 50mmHg indicated that there was a leak in the circuit and sutures needed to be examined before the preparation could continue. Secondly, the viability of the nerve contained within the recording electrode was also examined. If the nerve failed to elicit a typical reproducible response to distension (**fig 2.5.2**), which is most likely due to damage during the dissection process, then this nerve bundle was discarded and a new bundle was inserted into the electrode. After allowing the preparation to stabilise for a period of 30 minutes with no volume in the bladder, repeat control distensions with isotonic saline (NaCl 0.9%) were performed. To perform control distensions, the outflow tap was closed and the syringe driver was set to fill the bladder at either 30 µl/min or 100µl/min to a final intravesical pressure of 30-40mmHg every 10 minutes, after which, the outflow tap was opened and the bladder was allowed to empty. This was repeated until a stable pressure and afferent response to bladder distensions was obtained.

In order to examine the physiology of the bladder, a number of pharmacological agents were applied either directly to the outside of the bladder and thus were able to bind the receptors of both the muscle and the nerve bundle, or into the lumen of the bladder via the syringe driver. The precise protocols relating to the following applications will be explained in further detail within each chapter but the general protocols will be explained here:

Intraluminal application of pharmacological agents

Pharmacological agents were applied to the urothelial surface of the bladder through the syringe driver catheter entering via the urethra. All pharmacological agents were diluted in isotonic saline and placed in syringes on the syringe pump in place of isotonic saline.

These pharmacological agents were applied in two distinct ways and will be clarified for specific experiments: either with the outflow tap remained open so the drug passes freely through the bladder at a rate of 30µl/min, causing no distension and thus no effect on intravesical pressure, or were used in place of isotonic saline to perform bladder distentions.

Changes in afferent nerve activity were recorded during infusion of the drug as well as pressure and afferent nerve activity during or after distensions in the presence of the drug.

Extraluminal application of pharmacological agents

To examine both bladder afferent nerve and detrusor muscle responses to the application of pharmacological agents, these drugs were applied directly to the extraluminal surface of the bladder. This was performed using two different protocols depending on whether the drug was an agonist or an antagonist and the pharmacological profile of the drugs being used. In the case of antagonists, pharmacological agents were perfused in the extraluminal krebs.

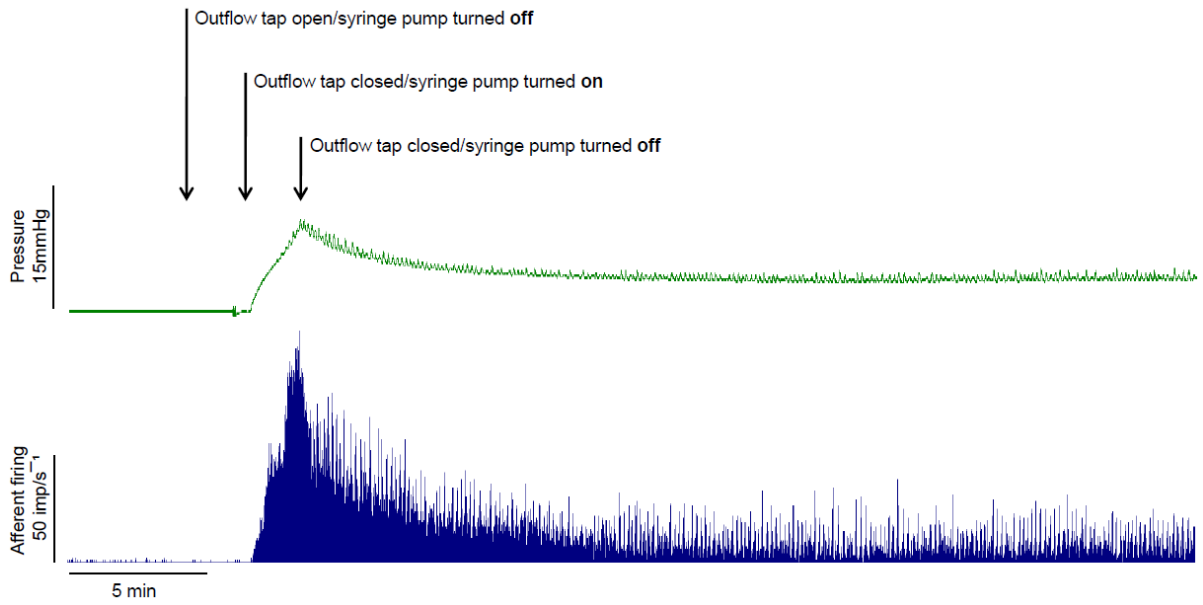

Fig 2.4.1 *Experimental trace showing stabilisation of detrusor smooth muscle and afferent nerve activity following distension to 12mmHg. The Intravesical pressure, and corresponding afferent nerve discharge gradually becomes stable in the presence of a set volume after approximately 30minutes.*

In many experiments, in order to measure both afferent nerve and intravesical pressure responses to pharmacological agents, the bladder was filled to an intravesical pressure of around 12mmHg above baseline at a rate of 30ul/min. The infusion of saline was stopped but the outflow tap remained closed, and thus maintaining some volume within the bladder. Following a period of 45

minutes, which allowed the bladder muscle and neuronal firing to stabilise (**fig 2.4.1**), agonists were applied as a bolus dose from a pipette directly into the bath. Changes in afferent nerve activity were recorded during perfusion of the drug as well as pressure and afferent nerve activity during or after distensions with the drug.

Isolation and culture of primary urothelial cells

The method for the isolation of urothelial cells from the mouse bladder was derived from a number of previous studies (Birder, Nakamura et al. 2002; Everaerts, Vriens et al. 2010) and optimised for use in our laboratory. Following cervical dislocation, bladders were excised from the mouse and placed in phosphate buffered saline (PBS) (Gibco®), before the urethra and any excess tissue from the serosal side was removed. The bladders were cut longitudinally from the urethral opening and pinned flat with the urothelial side facing upwards on a Sylgard® (Dow Corning) coated dish. The bladder was washed 3 times in fresh Modified Eagle Medium (MEM) (Gibco®) containing 1% Antibiotic-Antimycotic (PSF) solution (Gibco®) and 0.7% HEPES. Bladders were then incubated with 2.5mg/ml Dispase in MEM for 2hrs at room temperature in the tissue culture hood. The dispase was aspirated and the urothelial side of the bladder was gently scraped with a blunt scalpel under a dissecting microscope. Removed urothelial cells were immediately placed into 0.5% trypsin-EDTA (Gibco®) and then incubated at 37°C for 5-10 minutes or until fully dissociated with gentle trituration every few minutes. The trypsin-EDTA was deactivated by addition to MEM with 10% Fetal calf serum (FCS) (Gibco®). The cell suspension was then spun at 1500rpm for 15min at 4°C. Media was aspirated and the pellet was resuspended in Keratinocyte serum-free media (K-SFM) (Gibco®). Cells were washed through a spin at 1500rpm for 15min at 4°C. The media was aspirated and the pellet resuspended in fresh K-SFM. The cell suspension was counted and plated on collagen (IV) (Sigma-Aldrich Poole,UK) coated coverslips and incubated overnight at 37°C in an atmosphere of 5% CO_2- 95% O_2 for use in both calcium imaging and immunohistochemistry experiments.

Isolation and culture of Dorsal root ganglion neurons

After cervical dislocation, whole DRG's from lumbosacral (L6/S2) and thoracolumbar (T11/L2) regions were removed. DRG's were immediately placed in Hanks Balanced Salt Solution (HBSS) (pH 7.4) (Gibco®) and treated with 2mg/ml papain (Sigma- Aldrich (Poole,UK)) and 0.5mg/ml cystein

(Sigma- Aldrich (Poole,UK)) and left to incubate at 37°C for 20 minutes whilst being gently agitated before being replaced by HBSS containing 4mg/ml collagenase (Sigma- Aldrich (Poole,UK)) and 5mg/ml dispase (Sigma- Aldrich (Poole,UK)) and left to incubate at 37°C for 20 minutes whilst being gently agitated. Following enzyme treatment, the DRGs were washed with fresh DMEM-F12 (Invitrogen) culture media containing 10% fetal bovine serum (FBS) (Gibco®) before being resuspended in 500µl of fresh DMEM-F12 media. The DRGs were then triturated with a Pasteur pipette to release individual DRG neurons, which were counted, and plated onto Matrigel (Sigma-Aldrich (Poole,UK)) coated glass coverslips (6 x 16mm). Cells were then incubated in a 5% CO_2-95% O_2 incubator at 37°C for 2 hours. Following incubation, the cells were flooded with DMEM-F12 (1% PenStrep, 10% FBS) culture media (Invitrogen) and incubated overnight at 37C in a 5% CO_2-95% O_2 incubator for use in calcium imaging experiments.

Calcium imaging

Following a 24hr incubation at 37°C, cells attached to coverslips were incubated with 2µM Fura 2 acetoxymethyl ester (Fura2-AM) (Sigma- Aldrich (Poole,UK)) for 30 min at 37°C in the dark. Fura2-AM is ratiometric fluorescent dye which diffuses into cells and binds to free intracellular calcium. Coverslips were then placed in a washing well containing only media at 37°C before being washed in HEPES solution at room temperature and then placed upon the recording equipment. Coverslip containing cells were then transferred to a perfusion chamber and mounted on an Epifluorescent microscope where they were continually perfused with HEPES buffer at a rate of 1ml/min for 30 minutes to allow excess Fura2-AM to wash from the cells. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as the ratio between the fluorescence signal emitted following a 100ms^{-1} excitation at 340/350 nm and at 380 nm light. A region of interest (ROI) was placed around all individual cells visible within the single view field as well as a blank region where no cells were present to provide a baseline light emission reading. After 30 minutes of perfusion with HEPES buffer, cells were stimulated for 2-5 minutes with HEPES containing the drug of interest. The drug was then washed out by switching the perfusion back to HEPES buffer, finally the cells were stimulated with the calcium ionophore ionomycin (5µM) (Sigma- Aldrich (Poole,UK)). Ionomycin was applied as a positive control and only positively responding cells were included in the analysis. All calcium imaging was performed at room temperature as a heated perfusion system was not available. After correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation

wavelengths was calculated and results expressed as a ratio of this fluorescence (Rf). For each experimental condition, at least 15 cells in at least three independent experiments were assayed.

Buffers

HEPES buffer for DRGs; composition in mM (NaCl 134, KCL 5, HEPES 9.4, glucose 10, $CaCl_2$ 2, $MgCl_2$ 1).

HEPES buffer for urothelial cells; composition in mM (NaCl 142, $NaHCO_3$ 5, HEPES 10, glucose 16, KCL 2, $CaCl_2$ 2, $MgCl_2$ 1, 0.1% bovine serum albumin) (Sigma- Aldrich, Poole, UK) added to the dissolved solution.

Calcium free HEPES buffer for urothelial cells: composition in mM (NaCl 142, $NaHCO_3$ 5, HEPES 10, glucose 16, KCL 2, $MgCl_2$ 3, 0.1% bovine serum albumin added to the dissolved solution.

All solutions were adjusted to pH 7.4 with NaOH and had an osmolarity of 300-310mosmol/L to provide isotonic conditions.

Immunohistochemistry

Following the culture of urothelial cells for 24hrs on collagen (IV) coated coverslips, surrounding culture media was removed and the cells were washed three times with sterile PBS. PBS was aspirated and the cells were fixed with 1ml/well cold paraformaldehyde (PFA) (4%) (Sigma- Aldrich (Poole,UK)) and left in the fridge for 45 minutes. PFA was removed and the cells were permeabilised with 4°C sapponin 0.05% + 2% FBS prepared in PBS (1ml/well) and incubated at 4°C for 45 minutes. Following permeabilisation, the sapponin was removed. The primary antibody was prepared in saponin +FBS (1/50) and 50µl was applied to each coverslip before being left in the fridge overnight. After 24hrs, excess primary antibody was removed with PBS washing (X3). The secondary antibody was prepared in PBS + FBS 2% (1/1000) and 50ul was applied to each coverslip and left at room temperature in the dark for 2 hours and then washed with PBS (X3). The coverslips were then mounted on slides using Vectashield® mounting media (containing Dapi) (Vector Laboratories, UK) before being left to dry in the dark and then imaged using a fluorescent microscope.

Primary antibody for urothelial cells was 1/50 Cytokeratin 7 Clone OV-TL 12/30 monoclonal mouse anti-cytokeratin 7 (DAKO) raised in mouse.

Secondary antibody used for urothelial cells was 1/1000 Alexa Fluor® 488 green goat anti-mouse (Invitrogen) raised in goat.

RT-qPCR

Extraction of mRNA from PMUC and DRG neurons

Following isolation of PMUC and DRG neurons from the mouse as per methods described above, cells were incubated at 37°C in respective media for 24hrs, all RNA extractions were performed at room temperature and following the manufacturer's instructions for the RNeasy mini kit (Qiagen, Australia). Lysis of the cells was performed by addition of buffer RLT (350µl) (1% β-Mercaptoethanol) to the cell pellet before being passed repeatedly through a needle (19G) attached to a sterile syringe. Ethanol (70%) (350µl) was added to the lysate and mixed by pipetting before transfer to a spin column. Following centrifugation, the flow through was discarded and the RNA was now contained within the spin column. Further purification of the RNA contained within the spin column was achieved through 15s cycles at 10,000rpm with both RW1 and RPE buffers. To elute the RNA, 30µl of RNase free water heated to 42°C was added directly to the spin column membrane and centrifuged for 1 minute at 10,000rpm. The eluted RNA was then used to repeat this process, ensuring the highest possible concentration of RNA in the collection tube.

RNA was stored at -80°C to prevent decomposition and was all converted to cDNA at the earliest possible time point to prevent freeze thaw cycles.

Purity and integrity of the RNA sample

A NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the total RNA quantity and to assess the purity. Purity of the RNA was determined based on the 260/280 and 260/230 ratios' of absorbance. A 260/280 absorbance in the range of 2±0.1 and a 260/230 absorbance in the range of 2.0-2.2 was considered acceptable and this RNA was then used to synthesise cDNA for use in qPCR reactions.

Total mRNA extraction values from individual mice varied due to the individual variation in size between bladders of different mice. RNA extraction from urothelial cells of a single mouse ranged from 25-35ng/µL. As such, the point at which mRNA was converted to cDNA provided an opportunity

to more easily standardise the quantity of cDNA produced by adding the same quantity (ng) of RNA to the cDNA synthesis kit and was standardised at 220ng.

Synthesis of cDNA

Synthesis of cDNA from RNA was performed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Superscript III is a high performance propriety type of reverse transcriptase which is optimised for the synthesis of first-strand cDNA from total RNA as it is not significantly inhibited by ribosomal and transfer RNA. For each reaction, 10 µl of 2X reverse transcriptase reaction mix (which also contains an RNase inhibitor protein) was added to 2µl of reverse transcriptase enzyme mix, containing: oligo$(dT)_{20}$ (2.5µM), random hexamers (2.5ng/µl), $MgCl_2$ (10mM) and dNTPs, and then made up to a total of 20µl with a combination of RNA and DEPC-treated water. The proportion of RNA/H_2O was dependent on the concentration of the RNA used, with a total of 220ng of RNA from urothelial cells used in each reaction.

A much larger amount of mRNA was extracted from mouse DRG neurons than urothelium, in the range of 190-230ng/µl total RNA. As such, the RNA was diluted by a factor of 1/10 so that similar amounts of total mRNA to that obtained for PMUC were used for the conversion to cDNA.

Following the manufacturer's instructions, the tube contents were gently mixed and incubated at 25°C for 10 minutes, 50°C for 30 minutes, and then terminated by heating to 85°C for 5 minutes before being immediately chilled on ice. 1µl of E. Coli RNase H was added to the cDNA for 20 minutes at 37°C before being stored at -80°C for use in qPCR reactions.

Quantitative real-time polymerase chain reaction (qPCR)

An iQSYBR Green Super Mix (BioRad) was used to amplify the cDNA target genes in the qPCR reactions. This supermix includes the SYBR green dye, which binds to the double stranded DNA as it is generated by the PCR reaction and allows the product to be quantified. qPCR was performed using a Research Rotor-Gene 6000 real time thermocycler (Corbett-Qiagen). The PCR reactions were made up to a total of 25µl in each individual 100µl PCR tube (Corbett) and were prepared on -20°C pre-cooled metal tube carriers. Each PCR tube contained 12.5µl of the iQSYBR Green master mix, 2µl of primers (1µl each of forward and reverse) and a mix of cDNA and DEPC treated H_2O to make up to 25µl. Cyclic conditions were set at: 95°C for 12 minutes as an initial hold stage followed by 40 cycles

of 95°C for 30s, 59°C for 30s, 72°C for 30s, followed by a melt curve of 0.5°C increments every 30 seconds from 72-95°C. The number of cycles required to the point that the specific PCR product is amplified in a linear way is referred to as the cycle threshold (CT). This is the representation used for quantitative analysis, where a single CT difference (Δ CT = 1) represents a two-fold difference in the quantity of the target gene. Therefore, after each qPCR reaction, the CT value at linearity was determined for each gene and is a representation of relative expression levels of that particular gene. Expression levels of each target gene were expressed relative to the housekeeping gene, β-actin, and in the case of purinergic receptors, represented as a ratio relative to $P2Y_1$ expression.

Controls were performed in which no cDNA was added to the reaction mix.

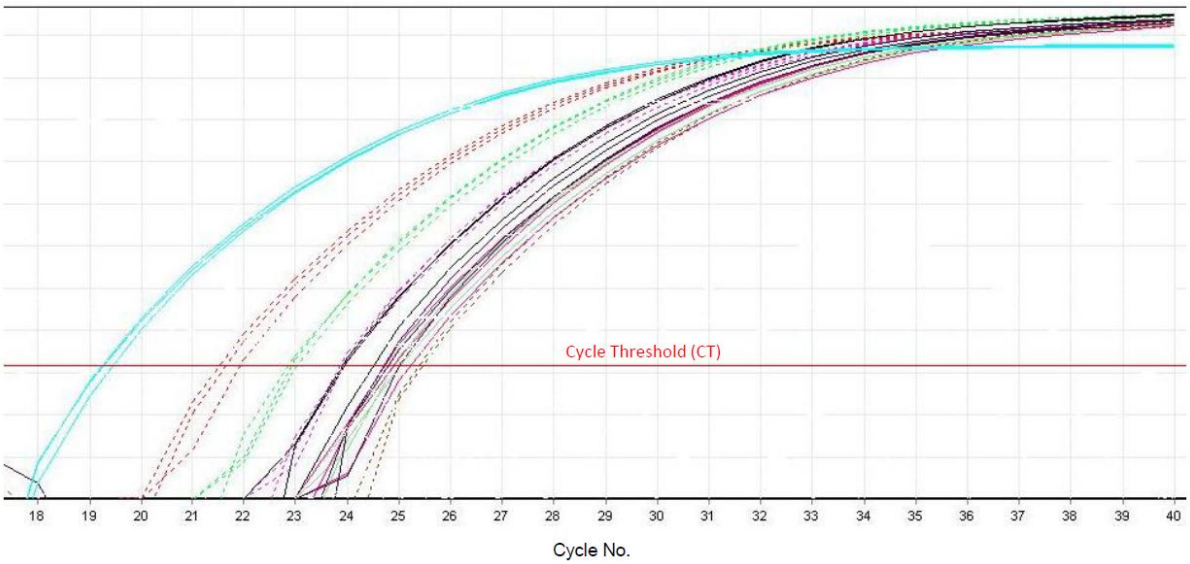

Fig 2.4.2 *Example trace of gene amplification during qPCR reactions. The CT is set to the linear phase of expression levels.*

Primer design and selection

Oligonucleotide primers were designed from the characterised mouse genome (mus musculus). The primers (**table 2.4.1**) were designed following a strict set of rules ensuring specificity of the primers to the gene of interest and purchased from Geneworks (Thebarton, South Australia).

Gene		Primer Sequence '5 – 3'	Product	Position	Tm°C	Chromosome
$P2X_1$	Forward	CAAGTATGCGGAGGACATGG	131bp	1362-	58.4	11
	Reverse	CACACTGAGTCAAGTCCGG		1493	57.8	
$P2X_2$	Forward	CCATCAGGTGAAGGACCAGC	118bp	1454-	60.4	5
	Reverse	GCTGGTCAAGAGTGTCCACC		1571	60.6	
$P2X_3$	Forward	CAGTGTTCACCAGTGACCAGGC	128bp	1086-	63.6	2
	Reverse	GCCTGTTGAAGGTTCTGCAGCC		1213	64.4	
$P2X_4$	Forward	CGTCTGTCACTCTAGAGACGG	202bp	1229-	59.3	5
	Reverse	GGTGCTGTTATGGACGTGTGG		431	61.5	
$P2X_5$	Forward	GTAGCCAGAGCTCTTGGCAGG	115bp	1494-	62.8	11
	Reverse	CTCAGAAGCCACATCCTGAGC		1608	60.8	
$P2X_6$	Forward	GACCTGCTGCTACTGTATGTGG	104bp	1120-	60.7	16
	Reverse	GGCTCGGTCTATGAACTGTTGG		1226	61	
$P2X_7$	Forward	CACATTCGCATGGTGGACCAGC	98bp	1213-	64.5	5
	Reverse	GACAGGTCGGAGAAGTCCATCTGG		1310	64.3	
$P2Y_1$	Forward	GTCTCAACAGCTGTGTGGACC	206bp	1598-	61	3
	Reverse	CTCAGGAGCTAGGATCTCGTGC		1803	62	
$P2Y_2$	Forward	CACGATGGACTTAGCTCAGAGG	207bp	1864-	60.2	7
	Reverse	CAGGAGGCAGAGATAACAGGC		2070	60.2	
$P2Y_4$	Forward	CAGCAGCTATGCAGAGGTAGC	194bp	553-	60.9	X
	Reverse	CCTCTGCCTGCAGTTAGTCC		746	60.1	
$P2Y_6$	Forward	GGCAACTGGTCAATTCATGC	150bp	1583-	58	7
	Reverse	CACATCCTGAGATGTCTAGC		1732	55	
$P2Y_{12}$	Forward	GTGTCAACACCACCTCAGCC	149bp	420-	61.1	3
	Reverse	CCTCATTGCCAAGCTGTTCG		568	59.8	
$P2Y_{13}$	Forward	GTTCCTCAAGATCATCATGCCG	210bp	436-	59.4	3
	Reverse	GTGTGACTGACCACCTGATGC		645	61.2	
$P2Y_{14}$	Forward	GTCACGAAGATACAGTGCATGG	131bp	792-	59.4	3
	Reverse	GTGATGGCCGTGTAGAAGACG		922	61	
NK1	Forward	GACACTTAGTCTGCCAAGAGC	193bp	3561-	58.7	6
	Reverse	CTCCACATGCTGGATAGAGC		3753	57.8	
NK2	Forward	GGATGGTGACATGGCTCAGC	117bp	1879-	61.1	10
	Reverse	GGACAGCTTGCAGACGTTGG		1995	61.8	
NK3	Forward	CCTCATTCTCCTAACCATGCC	205bp	1681-	58.1	3
	Reverse	CAGACACCGGATACTTAGAGC		1885	57.6	
TRPV1	Forward	CAAGGCTCTATGATCGCAGG	198bp	575-	58.2	11
	Reverse	GAGCAATGGTGTCGTTCTGC		772	59.8	

Table 2.4.1 *Primer sequence, product size, gene loci, and Tm for all genes for which RT-qPCR was performed.*

The mouse genes were identified within unigene through their specific receptor nomenclature. For design of the purinergic receptor genes especially, but also for the tachykinin receptors and the TRPV1 receptor, it was essential to ensure that these primers were specific for that receptor as a large portion of the desired gene is conserved between each of the different receptor isoforms. As

such, exon regions were blasted against all other isoforms to identify regions that were specific to each receptor before attempting to design the primers (**table 2.4.1**). All primers were designed within exons and were intron spanning.

Urothelium specific information

For quantifying gene expression in primary mouse urothelial cells a total of 4µl cDNA @ 1/50 dilution in DEPC H_20 was used and provided linear CT readings within the range of 20-30 PCR cycles. Samples were performed in triplicate and statistical analysis was performed to ensure the standard deviation was less than 0.16 before accepting results.

DRG specific information

Following transcription of DRG cDNA, it was diluted 1/50 with DEPC H_2O before being used in qPCR experiments. 4µl cDNA @ 1/50 dilution was used to provide linear CT readings of the target genes within the range of 20-30 PCR cycles. Samples were performed in triplicate and statistical analysis was performed to ensure the standard deviation was less than 0.16 before accepting results.

Urothelial release factors

The release of acetylcholine (ACh), ATP and prostaglandin E_2 (PGE_2) was examined using an isolated whole bladder preparation. The bladder was catheterised as described previously for afferent nerve activity so that intravesical pressure could be measured and the bladder could be filled and emptied accordingly. Bladders were constantly perfused at a rate of 30µl/min with the outflow tap open and situated in an eppendorf tube in dry-ice for 7 minutes, thus allowing a baseline sample of intravesical saline to be collected without stretching of the urothelial layers of the bladder. Following collection of a baseline sample, the outflow tap was closed and bladders were filled at a rate of either 30µl/min or 100µl/min to a maximal pressure of 40mmHg before perfusion was stopped and the outflow tap was open. The tube supplying the outflow tap was removed and placed into an eppendorf tube sitting in dry-ice and thus the maximal amount of bladder content would enter the eppendorf and be snap frozen to prevent enzymatic degredation within the intravesical supernatant. Urothelial mediators were measured both at rest and during stretch in $TRPV1^{-/-}$ (KO) and $TRPV1^{+/+}$ (WT) mice and with the infusion of NKA (300nM) dissolved in saline.

The ACh, ATP and PGE_2 assays were performed according to the manufacturers' protocols. Mediator concentration in samples was calculated using standard curves constructed from known ACh, ATP or PGE_2 standards and each plate included a blank which was subtracted prior to analysis.

ATP determination

Concentrations of ATP release from the bladder lumen were measured using a luminescence based ATP Determination Kit (Molecular Probes). The assay is based on luciferases' requirement for ATP in producing light from the reaction and thus gives a precise reading of the ATP present in the sample at the time of the reaction but will not pick up any previously broken down ATP.

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow[\text{luciferase}]{Mg^{2+}} \text{oxyluciferin} + \text{AMP} + \text{pyrophosphate} + CO_2 + \text{light}$$

Luminescence was measured using a Modulus Microplate Mulitimode Reader (Turner Biosystems, California, USA) and ATP content in samples was calculated from a standard curve of luminescence plotted using known concentrations of ATP (**fig 2.4.3**).

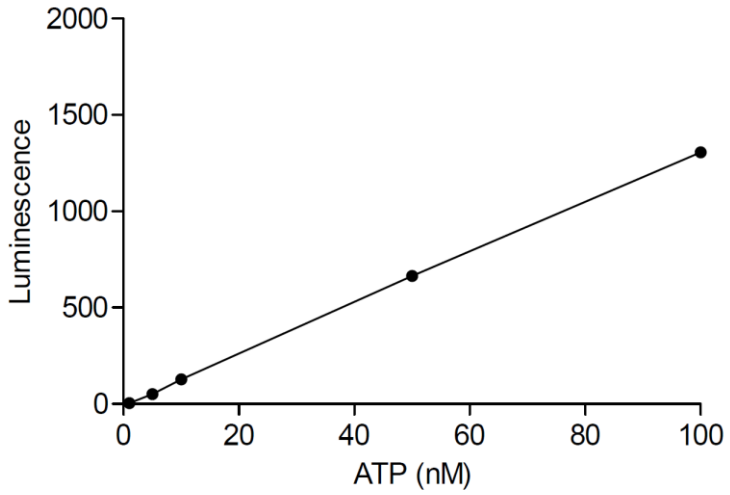

Fig 2.4.3 *Standard curve of luminescence readings to known concentrations of ATP*

Acetylcholine determination

Concentration of acetylcholine release from the bladder lumen was measured with an Amplex Red ACh/ACh esterase Assay Kit (Molecular Probes). First acetylcholinesterase converts acetylcholine to choline. Choline is in turn oxidised to betaine and hydrogen peroxide, which in the presence of horseradish peroxidise reacts with amplex red to generate the fluorescent product resorufin. Resorufin fluorescence is measured using a Modulus Microplate Mulitmode Reader (Em 590nm) (Turner Biosystems, California, USA). As the assay measures the metabolite choline, the assay not only calculates the current amount of acetylcholine, but also any acetylcholine that has been broken down during the course of sample collection.

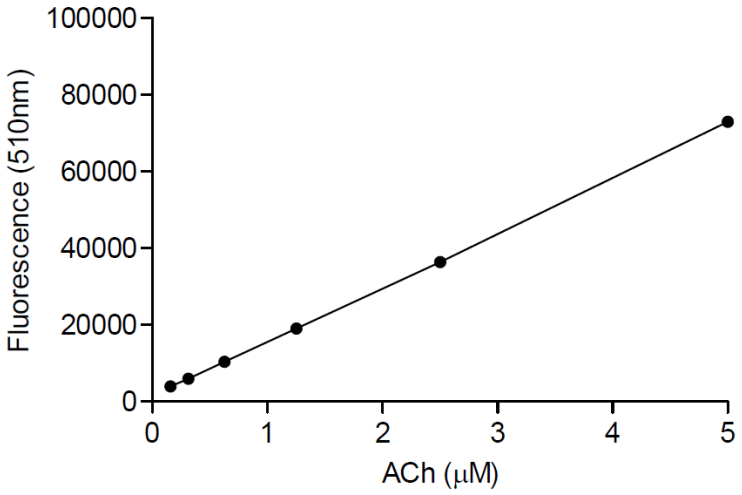

Fig 2.4.4 *Standard curve of fluorescence readings to known concentrations of ACh.*

PGE_2 determination

Concentration of PGE_2 was measured using Prostaglandin E_2 EIA Kit (Cayman Chemicals). The assay uses a 96 well plate coated with goat polyclonal anti-mouse IgG antibodies to which mouse IgG PGE_2 monoclonal antibodies are added. The assay then works by competitive binding between the PGE_2 in a sample and a PGE_2-acetylcholinesterase conjugate (PGE_2 Tracer). The binding of the PGE_2 Tracer is quantified by the addition of an acetylcholinesterase substrate leading to the production of a coloured substrate that absorbs strongly at 412nm. Samples and standards were analysed using a Modulus microplate reader (420nm) and the intensity of this colour determined is proportional to

the amount of PGE_2 Tracer bound to the well, which is inversely proportional to the amount of free PGE_2 present in the well during the incubation.

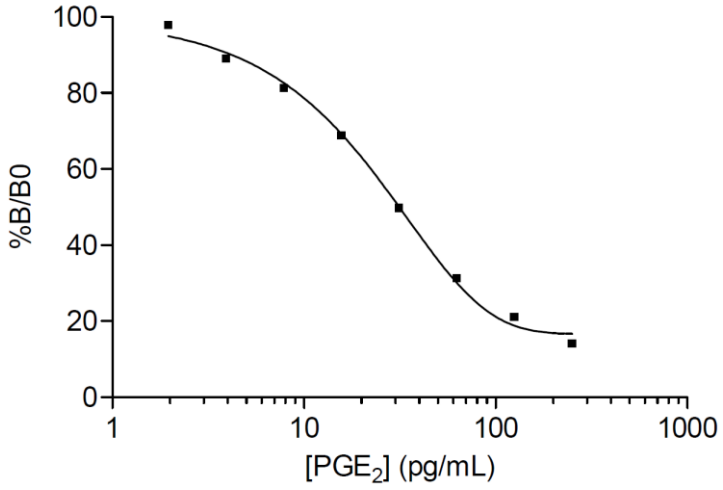

Fig 2.4.5 *Standard curve of spectrophotometer readings and known concentrations of PGE_2. Where B/B_0 is the standard bound/maximum bound.*

Drugs and Solubility

All the drugs used in this study are listed in the table below. All drugs were dissolved in dH_2O where possible, or in DMSO (Sigma), or 70% ethanol (Sigma) to make stock solutions. Stock solutions were either diluted in Krebs (for extraluminal application) or saline (for intraluminal application) to the final concentration required for individual experiments. For all experiments, a vehicle control was used to account for any effects of the solvent. Concentrations of pharmacological agents used in each study are described in the relevant experimental protocols section.

Compound	Main Action	Purchased from	Cat No.
α,β-Methyleneadenosine 5'triphosphate (αβMe-ATP)	P2X agonist	Sigma Aldrich	M6517
Adenosine 5'-triphosphate (ATP)	Purinergic agonist	Sigma Aldrich	A2383
Apyrase	ATPase enzyme	Sigma Aldrich	A6410
Capsaicin	TRPV1 agonist	Sigma Aldrich	M2028
Capsazepine (CPZ)	TRPV1 antagonist	Sigma Aldrich	C191
CP99994	NK1 antagonist	Tocris Bioscience	3417
1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP)	Nicotinic agonist	Sigma Aldrich	D5891
GR159897	NK2 antagonist	Tocris Bioscience	1274
Ionomycin	Calcium ionophore	Sigma Aldrich	I0634
Nifedipine	L-type Ca^{2+} channel blocker	Sigma Aldrich	N7634
NF449	P2X antagonist	Tocris Bioscience	1391
Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS)	P2X antagonist	Sigma Aldrich	P178
MRS2279	$P2Y_1$ antagonist	Tocris Bioscience	2158
MRS2365	$P2Y_1$ agonist	Tocris Bioscience	2157
MRS2578	$P2Y_6$ antagonist	Tocris Bioscience	2146
MRS2693	$P2Y_6$ agonist	Tocris Bioscience	2502
[Lys5,MeLeu9,Nle10]-NKA(4-10) (NKA)	NK2 agonist	Tocris Bioscience	3228
SB222200	NK3 antagonist	Tocris Bioscience	1393
[Sar9,Met(O2)11]-Substance P (Substance P)	NK1 agonist	Tocris Bioscience	1178
Senktide	NK3 agonist	Tocris Bioscience	1068
Tetrodotoxin (TTX)	Na^+ channel blocker	Tocris Bioscience	1078
Uridine 5'-triphosphate (UTP)	$P2Y_{2/4}$ agonist	Sigma Aldrich	U6875

Table 2.4.2 *Summary of the pharmacological agents used in this study*

2.5 CHARACTERISATION OF AFFERENT NERVE RESPONSES TO DISTENSION

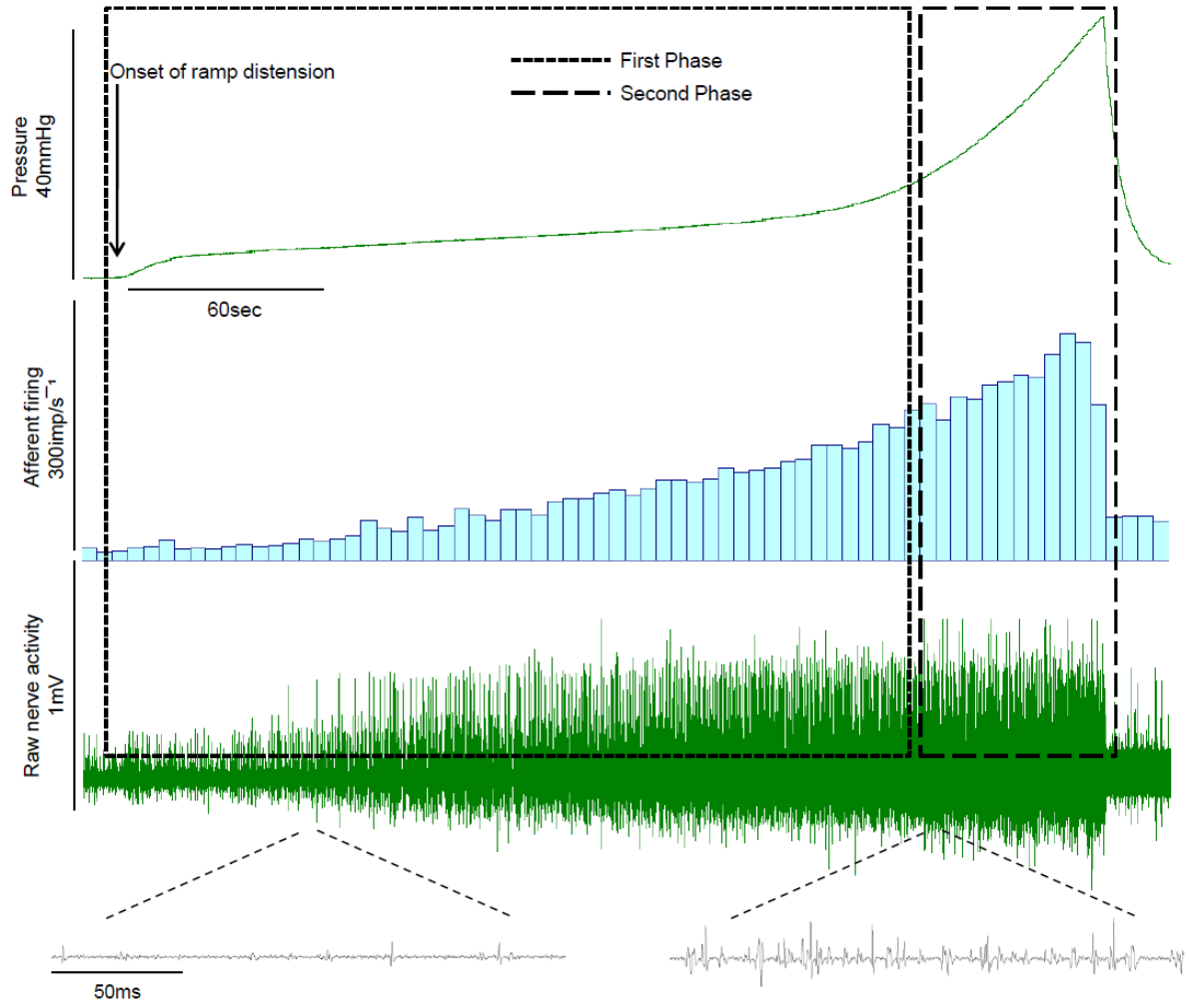

Fig 2.5.1 *The afferent nerve and pressure responses to bladder ramp distension.*

A*. The rise in intravesical pressure during ramp distension with isotonic saline (0.9%) at 30µl/min to a maximal intravesical pressure of 40mmHg.* ***B****. Sequential rate histogram portraying the increase in whole bladder afferent nerve discharge evoked during saline ramp distension.* ***C****. Raw nerve trace depicting the increased afferent activity in response to distension.*

In normal wildtype preparations, ramp distension provides a progressive increase in intravesical pressure and a simultaneous increase in afferent nerve activity (**fig 2.5.1**). In this study, bladders were either filled at a rate of 100µl/min or 30µl/min depending on the experimental protocol, but were never distended beyond a threshold of 40mmHg to prevent damage to the bladder wall. There were two distinct phases of the bladder response to an increase in volume. In the initial stage (0-10/15mmHg), there is large increase in volume with a small increase in pressure as the bladder muscle is able to accommodate the increase in intraluminal volume. A second stage occurred beyond 10-15mmHg in which there is a much larger increase in intravesical pressure for a relatively small increase in volume (**fig 2.5.1**).

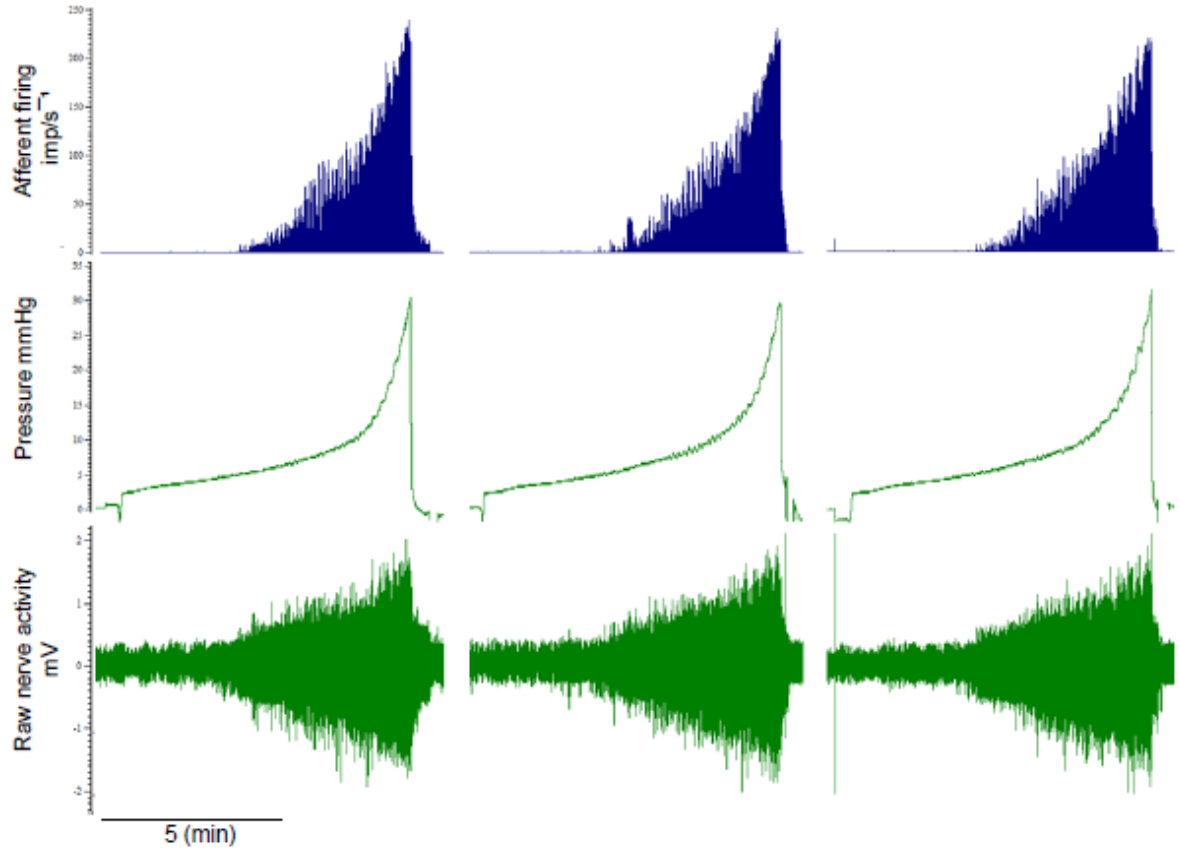

Fig 2.5.2. *Representative trace showing control ramp distensions carried out using isotonic saline at a filling rate of 30µl/min to a maximal pressure of 30mmHg. Distensions are performed at 10 minute intervals at the start of all experiments to confirm muscle and nerve viability. Nerve and muscle responses are stable and reproducible.*

The whole nerve afferent response to control distension was highly variable between preparations, but was very stable within a single preparation. The stability of the afferent response to repeat ramp distension was used as a positive control at the start of afferent nerve based experiments and an example trace can be seen in **fig 2.5.2**. The reproducibility of the afferent nerve and compliance responses to subsequent ramp distension can be seen in **fig 2.5.3**.

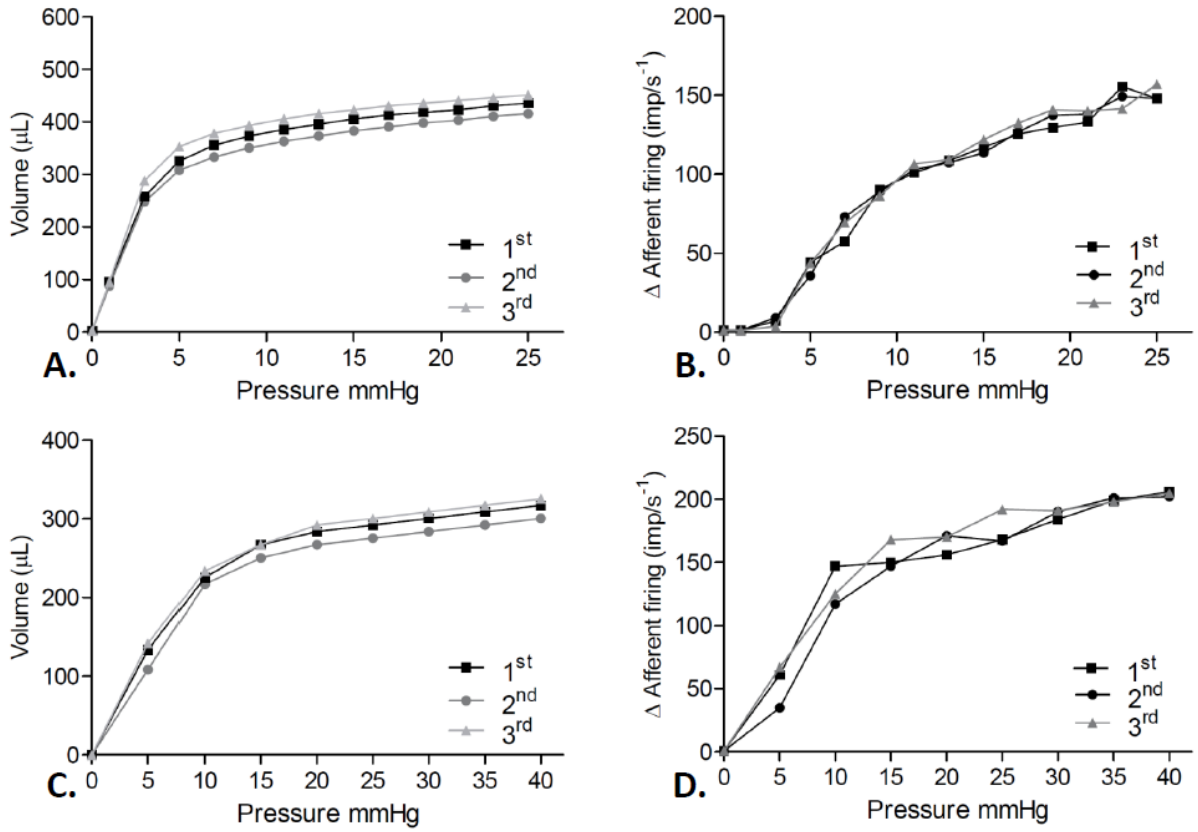

Fig 2.5.3 *Pressure/volume and pressure/nerve responses to three repeat distensions prior to the start of an experiment. The compliance and afferent response to distension are reproducible.* ***A****, pressure/volume relationship during ramp distension at 30µl/min.* ***B****, Afferent nerve/pressure relationship during ramp distension at 30µl/min.* ***C****, pressure/volume relationship during ramp distension at 100µl/min.* ***D****, afferent nerve/pressure relationship during ramp distension at 100µl/min.*

2.6 SPONTANEOUS BLADDER CONTRACTIONS

During ramp distension of the bladder with saline, transient increases in intravesical pressure are observed as overall pressure increases which are associated with bursts in afferent nerve firing. An example of this can be seen in **fig 2.6.1**. The development of spontaneous contractions during distension only occurs when the bladder is distended at a slower rate (30µl/min) and not at faster rate of 100µl/min. A closer look at these spontaneous contractions (**fig 2.6.2**) shows they exhibit phasic activity with a consistent amplitude and frequency within an experimental preparation.

Phasic spontaneous detrusor activity also develops when a bladder is distended to a physiological volume of 12mmHg and then left to accommodate the increase in volume (**fig 2.6.3**). These spontaneous contractions observe a similar frequency, but increased amplitude to those observed during bladder distension and are also associated with concomitant bursts of afferent discharge.

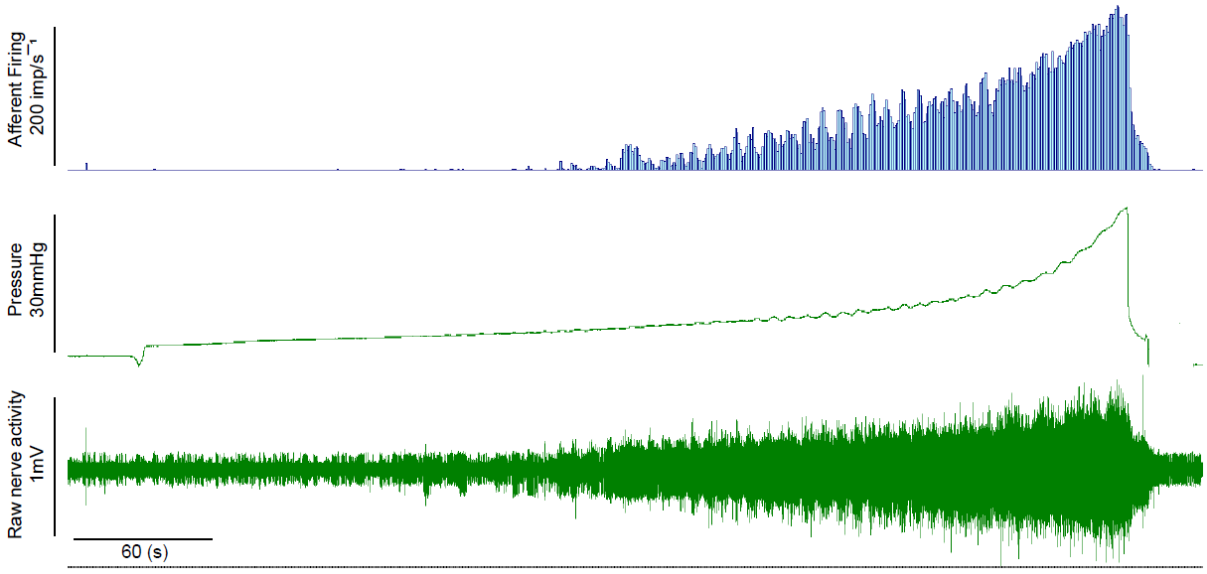

Fig 2.6.1. *Representative trace showing the spontaneous detrusor contractions which occur during control ramp distension carried out with isotonic saline at a filling rate of 30µl/min to a maximal pressure of 30mmHg. Bursts of afferent nerve activity can be seen within the raw nerve activity, corresponding to transient increases in bladder pressure.*

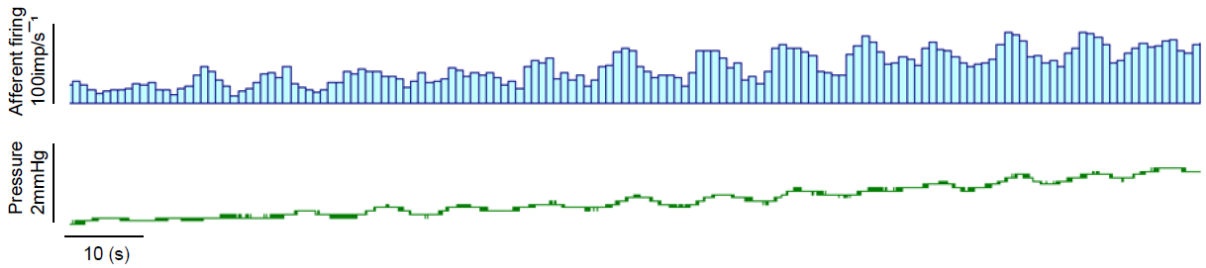

Fig 2.6.2 *A closer look at the interaction between spontaneous detrusor contraction and mean afferent discharge during ramp distension at 30µl/min.*

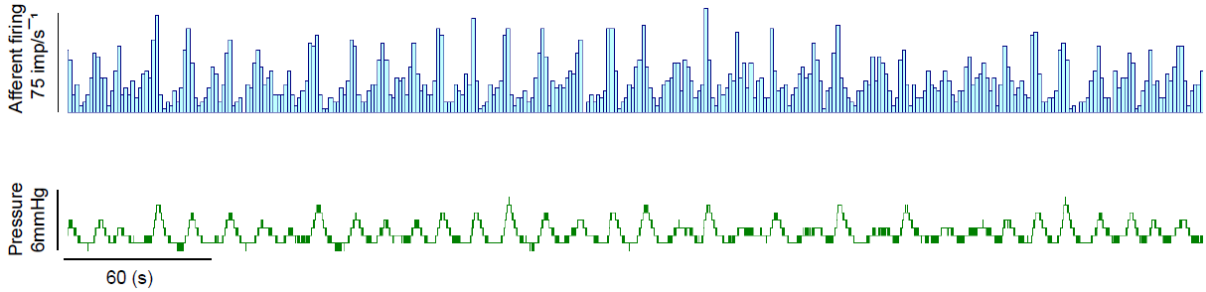

Fig 2.6.3 *Spontaneous detrusor contractions and associated afferent nerve activity occurring in a bladder distended to 12mmHg and left with saline inside.*

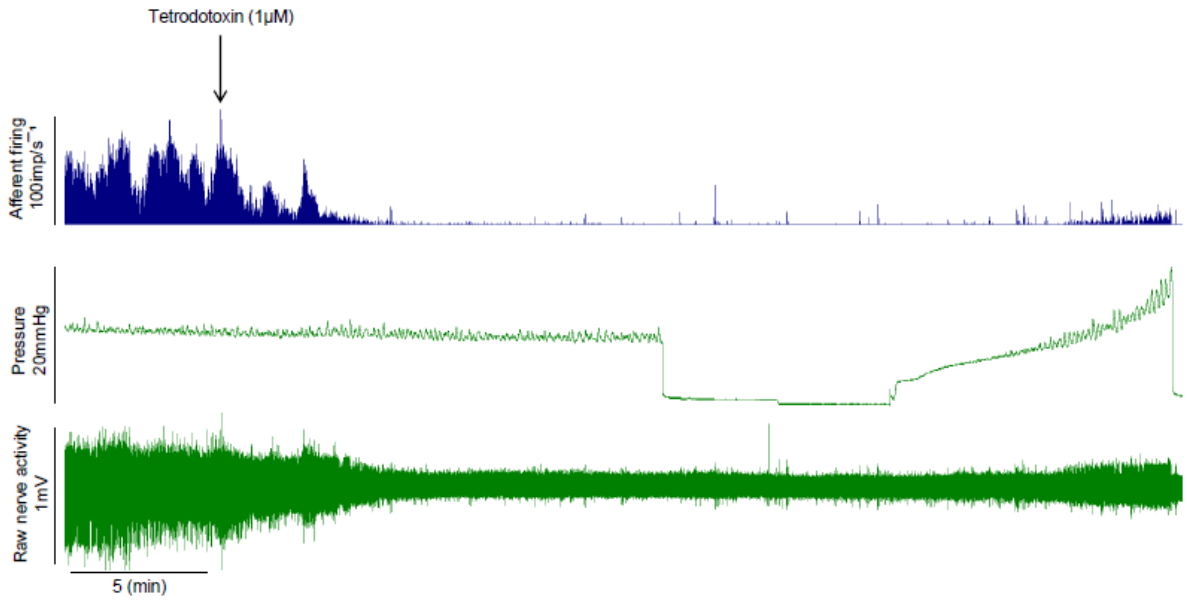

Fig 2.6.4. Representative trace showing *spontaneous detrusor contractions and associated afferent nerve activity occurring in a bladder pre-distended to 12mmHg before and after tetrodotoxin (1µM) followed by ramp distension with saline.*

As can be seen from the trace in **fig 2.6.4**, spontaneous detrusor contractions persist in the presence of tetrodotoxin (1µM). The afferent nerve response to bladder distension is abolished following the addition of tetrodotoxin to the bathing solution but has no effect on detrusor contractions when the bladder is pre-distended to a set volume or during ramp distension of the bladder at 30µl/min. This confirms the hypothesis that spontaneous detrusor contractions are not mediated by neuronal activity and appear to be myogenic in origin, potentially mediated by the interstitial cells in the sub-urothelium which are activated by a rise in intracellular Ca^{2+} and dependent on Cl^- currents (Sherrington 1892; Fry, Sui et al. 2007) as opposed to Na^+ currents. Spontaneous detrusor activity has previously been shown in the isolated whole bladder (Sherrington 1892) and more recent studies have shown a level of spontaneous contraction in the whole guinea pig bladder which were termed 'microtransients' (Drake, Harvey et al. 2003) and also shown to be tetrodotoxin insensitive. Prior to recent work elucidating the structural and electrical properties of interstitial cells, these were also proposed as the likely mediators of this phenomenon due to its myogenic origin (Gillespie 2004).

2.7 SPONTANEOUS AFFERENT FIRING

During most afferent nerve recording experiments there was a degree of spontaneous afferent nerve activity. An example of this can be seen in **fig 2.7.1**. In some experiments, approximately 2/3, large bursts of spontaneous activity are also observed following bladder distension which lasted for between 1-5 minutes (**fig 2.7.2**).

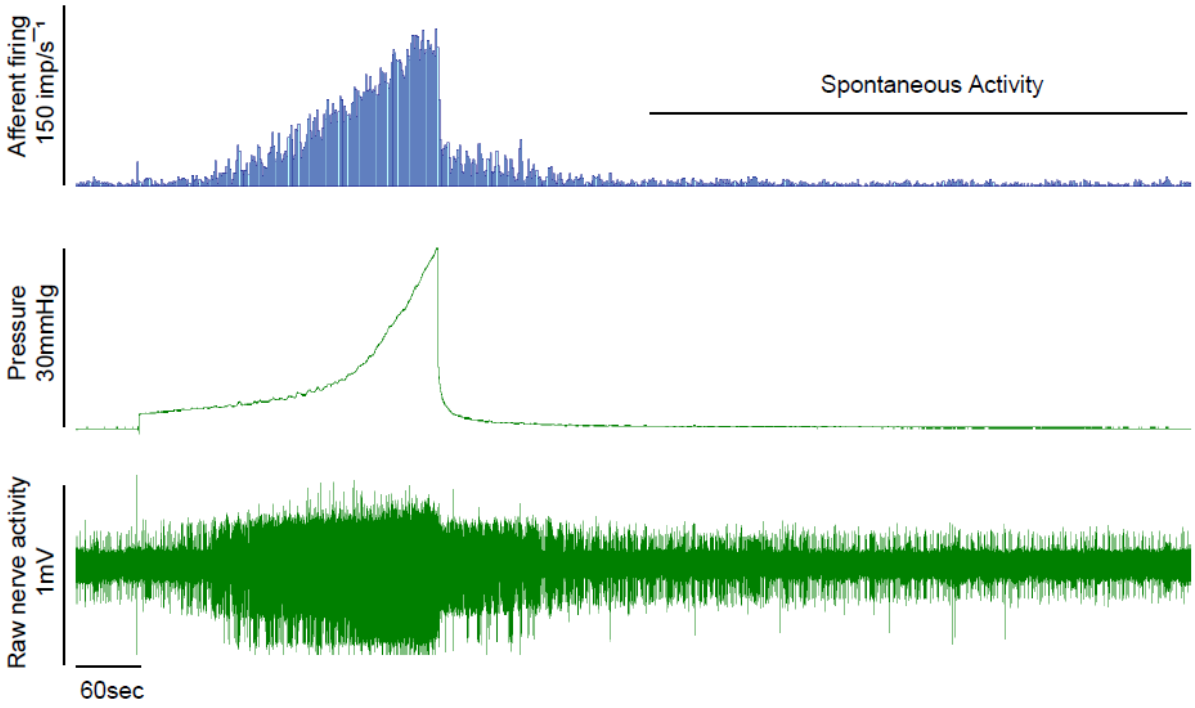

Fig 2.7.1 *Spontaneous afferent discharge in the absence of bladder distension.*

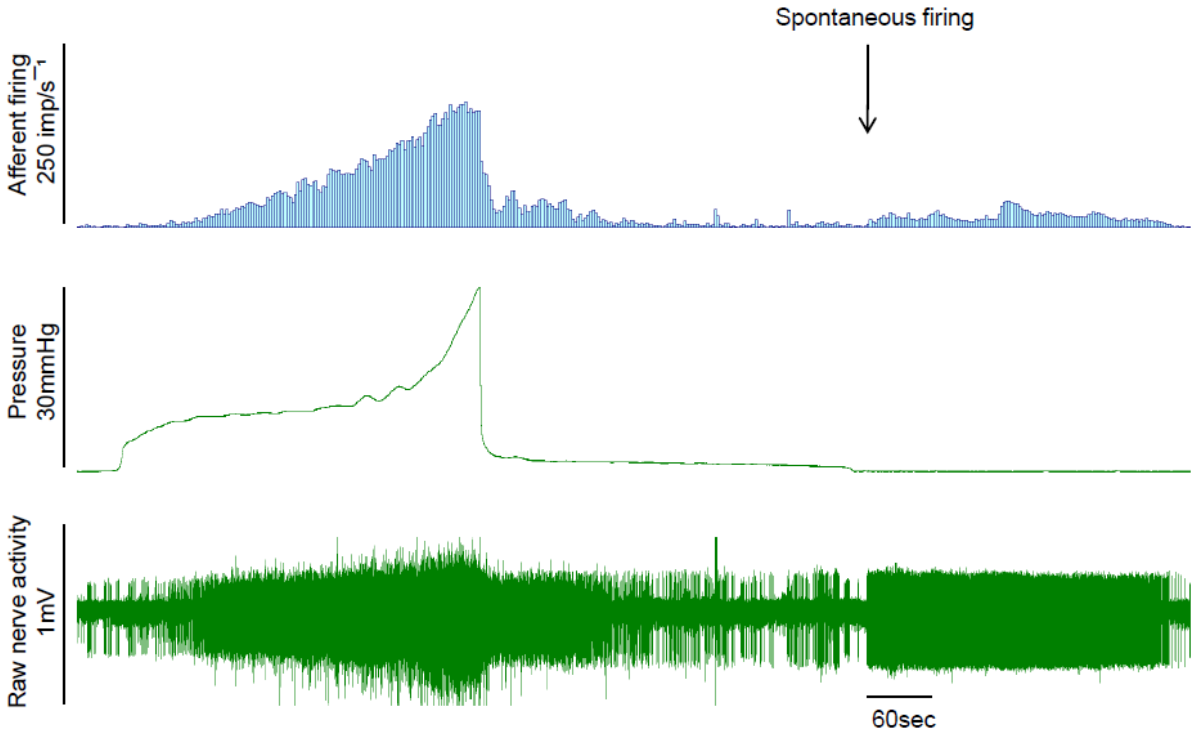

Fig.2.7.2 *Bursts of spontaneous afferent discharge following ramp distension.*

The spontaneous baseline firing represented in **fig 2.7.1** was recorded when the bladder was empty and therefore not subject to any exogenous mechanical stretch. This is consistent with a number of other studies which have reported baseline activity in in-vivo experiments in the rat (Sengupta and Gebhart 1994; Su, Sengupta et al. 1997) and mouse (Xu and Gebhart 2008), but is in contrast to Rong et al (2002) who reported only a small amount of baseline activity in vitro in the mouse and similar reports in the rat in vivo (Shea, Cai et al. 2000). In studies which have reported a greater degree of baseline firing, the bladder distension is performed to higher intravesical pressures of 80-100mmHg whereas studies in which ongoing activity was more sparce, distension was carried out to a much lower intravesical pressure (Shea, Cai et al. 2000; Rong, Spyer et al. 2002). However, in the studies observed here, the distension pressure and the rate of bladder distension is lower still than the studies in the literature and thus seems an unlikely explanation. **Fig 2.7.2** represents spontaneous bursts in baseline firing following ramp distension of the bladder, the causes of which are unknown. It is possible that they could represent the activation of mucosal receptors responsive to light von-frey hair stimulation (Zagorodnyuk, Costa et al. 2006) responding to contact between mucosal surfaces following emptying of the bladder.

Above all else, these traces exemplify the importance of recording afferent nerve firing and intravesical pressure simultaneously as there are a number of subsets of afferent fibers within the bladder which can be discriminated by their activation characteristics. It is likely that bladder compliance is dependent upon the intrinsic properties of the detrusor and the urothelium and that the accommodation properties of the bladder wall are a major contributor to afferent nerve discharge and mechanosensitivity.

More details on experimental design will be given in a brief protocol section within each results chapter.

CHAPTER 3: PURINERGIC RECEPTORS OF THE UROTHELIUM

3.1 INTRODUCTION

ATP is an established neurotransmitter in both the peripheral and central nervous systems and exerts its diverse effects through actions on both fast acting ionotropic $P2X_{1-7}$ receptors, and G-protein coupled $P2Y_{1-14}$ receptors. Distinguishing P2X receptors is dependent on activation/desensitisation kinetics whilst P2Y receptors, although able to respond to ATP, also show a distinct rank order of potency for a range of endogenous stimuli, namely ADP, UTP and UDP (**table 1.7.1**). Ectonucleotidases, responsible for the metabolism of ATP molecules are also found in the suburothelial space and are considered to be essential in fulfilling the complement of adenosine based molecular actions within the bladder.

ATP actions in the bladder

As has been alluded to in many previous studies, ATP plays an essential role in the bladder, and in many cases it is considered to be a major stimulus for alterations in mechanosensitivity. Genetic deletion of specific purinergic receptors, namely $P2X_3$ and $P2X_{2/3}$, have been used to show the importance of ATP signalling within the bladder. Knockout mice show urinary bladder hyporeflexia, with decreased afferent nerve activity, decreased voiding frequency, and increased bladder capacity (Cockayne, Hamilton et al. 2000; Vlaskovska, Kasakov et al. 2001; Cockayne, Dunn et al. 2005). In similar preparations, also in mice, Rong et al found intravesical αβMe-ATP (a P2X selective agonist) increased multifiber discharge and reduced threshold for distension induced firing in high threshold C-fiber afferents (Rong, Spyer et al. 2002). These effects were attenuated by an ATP antagonist, which also significantly reduced responses in the absence of an exogenous agonist, implicating an endogenous source of ATP (Rong, Spyer et al. 2002). These results show that $P2X_3$ and $P2X_{2/3}$ receptors are functionally expressed on bladder sensory pathways which enhance afferent nerve activity in response to ramp distension as well as responding to exogenous P2X agonists. Importantly, a reduction in afferent activity in response to bladder distension was seen with P2X antagonism alone, further supporting a role for endogenous release of ATP. Furthermore, namasivayam et al showed that desensitisation or antagonism of purinergic receptors also significantly reduced afferent nerve responses in rats (Namasivayam, Eardley et al. 1999). Intravesical ATP and αβMe-ATP have been observed to induce bladder overactivity in conscious rats (Pandita and Andersson 2002), whilst intravesical infusion of purinoceptor antagonists suramin and PPADS can increase bladder capacity. Consistent with this role of purinergic receptors in regulating afferent nerve activity and

micturition is the presence of $P2X_3$ immunoreactive afferent nerves in the suburothelium of the bladder (Vlaskovska, Kasakov et al. 2001; Brady, Apostolidis et al. 2004; Apostolidis, Popat et al. 2005). In patients with neurogenic detrusor overactivity, a significant increase in $P2X_3$ immunoreactivity on afferent nerves has been reported (Brady, Apostolidis et al. 2004; Apostolidis, Popat et al. 2005). These novel studies also showed that those neurogenic detrusor overactivity patients whom responded in a urodynamically positive way to intravesical resiniferatoxin or BOTOX treatment, elicited a significant reduction in $P2X_3$ immunoreactive fibers to normal levels. Again, this presents evidence to suggest that $P2X_3$ immunoreactive afferents are not only important in normal bladder signalling, but may contribute to the manifestation of bladder disorders such as neurogenic detrusor overactivty.

Further evidence of ATP signalling in the bladder comes from purinergic mediated inward currents and large transient increases in intracellular Ca^{2+} in bladder interstitial cells (Sui, Wu et al. 2004; Sui, Wu et al. 2006), leading the author to propose that interstitial cells could act as an intermediary between ATP released from the urothelium and afferent nerve activation, or through known gap-junction coupling influence the detrusor smooth muscle cells more directly. A final proposed role for ATP within the bladder, over and above its role in parasympathetic mediated stimulation of detrusor smooth muscle, is the inducement and proliferation of inflammation through activation of urothelial P2Y receptors (Säve and Persson 2010; Kruse, Säve et al. 2012), and a subsequent increase in the release of pro-inflammatory cytokines including IL-8 and IL-6. These cytokines most likely play a role in maintaining sub-urothelial neuronal excitability, however, as will be described below, an increase in urothelial ATP release is observed in many bladder syndromes and the corresponding increase in pro-inflammatory cytokines could lead to neuronal sensitisation (Oprée and Kress 2000; Obreja, Schmelz et al. 2002; Viviani, Gardoni et al. 2007).

There is ample evidence to implicate the purinergic system in both normal and abnormal voiding reflexes but as the main stimulus for purinergic receptors is ATP, it can be assumed that release of this neurotransmitter from a local source is a pre-requisite to these effects.

Non-neuronal ATP release

Bladder distension, or stretch of the urothelial cell layer in isolation, has been shown to induce ATP release (Ferguson, Kennedy et al. 1997; Birder, Nakamura et al. 2002; Sadananda, Shang et al. 2009; Sadananda, Kao et al. 2012; Collins, Daly et al. 2013). Whereas the stimulus for ATP release from neurons is depolarisation of the pre-synaptic membrane and calcium mediated vesicular release, the

major stimulus for ATP release from non-neuronal urothelial cells has been shown to be stretch of the bladder wall. This was first shown through the use of isolated tissue mounted in Ussing chambers (Ferguson, Kennedy et al. 1997) but has since been shown in both whole bladder experiments exposed to ramp bladder distension (Collins, Daly et al. 2013), and mechanical stretch of isolated bladder strips (Sadananda, Shang et al. 2009; Sadananda, Kao et al. 2012). ATP release has also been observed in urothelial cell culture models using both hypotonic solutions (Birder, Barrick et al. 2003; Cheng, Mansfield et al. 2011) and hydrostatic pressure to evoke a mechanical response (Olsen, Stover et al. 2011). The mechanism underlying urothelial release of ATP has yet to be fully characterised and it has been proposed that there are distinct mechanisms for basal and stretch evoked release. The release of urothelial ATP through activation of mechanically-gated channels has not been determined since gadolinium and amiloride, agents which block stretch activated ENaC and ASIC channels, have been found to have inconsistent effects on ATP release (Knight, Bodin et al. 2002; Birder, Barrick et al. 2003; Sadananda, Shang et al. 2009). Du et al (2007) showed that intravesical perfusion of 1mM amiloride significantly attenuated ATP release and was proposed as the mechanism responsible for a reduced frequency in reflex voiding during cystometry. However, amiloride is also known to antagonise ASIC channels at the concentrations used (Alexander, Mathie et al. 2011) and gadolinium is known to inhibit the luciferin-luciferase ATP assay used in these studies (Boudreault and Grygorczyk 2002). Thus no firm conclusions regarding the mechanism of direct mechanosensitive ATP release can be drawn.

As with neuronal release of ATP, an essential role for calcium has been proposed in the urothelium. It was found that liberation of calcium from the endoplasmic reticulum induces significant ATP release, yet blockade of store-operated extracellular calcium entry significantly enhanced distension-induced ATP release (Matsumoto-Miyai, Kagase et al. 2011). Thus, stimulation of calcium release from the endoplasmic reticulum induces urothelial ATP release, as well as a subsequent inducement of store operated calcium entry which suppresses the amount of urothelial ATP released, and thus providing a self regulating negative feedback mechanism. This observation has since been corroborated by others who have shown that distension-induced ATP release is mediated by a rise in intracellular calcium (Dunning-Davies, Fry et al. 2013) and that BAPTA-AM or agents that interfere with internal calcium storage block ATP release (Birder, Barrick et al. 2003). Antagonism of the IP_3 receptor has also been shown to reduce distension-induced ATP release (Matsumoto-Miyai, Kagase et al. 2009). A significant role for calcium in urothelial ATP release is supported by considerable evidence for a vesicular release mechanism. Crucially, both monensin and brefeldin A, known blockers of vesicle formation and trafficking, inhibited calcium-dependent distension evoked ATP release (Knight, Bodin et al. 2002; Birder, Barrick et al. 2003). Botulinumtoxin inhibits vesicular exocytosis via cleavage of

SNAP-25 and has since been shown to significantly reduce distension evoked ATP release (Smith, Vemulakonda et al. 2005; Collins, Daly et al. 2013; Hanna-Mitchell, Wolf-Johnston et al. 2013), further indicating that vesicular exocytosis is essential for ATP release. Another school of thought relating to vesicular release mechanisms is that it is an increase in hydrostatic pressure that initiates discoidal/fusiform vesical exocytosis of ATP during membrane insertion, and that activation of purinergic receptors on urothelial cells plays a role in membrane trafficking via calcium signalling pathways (Wang, Lee et al. 2005) in order to maintain the barrier function of the urothelium.

The importance of ATP release has been indicated by alterations in the levels of distension evoked release in disease and a significant increase in age (Yoshida, Homma et al. 2001), a known risk factor for all lower urinary tract symptoms. In models of spinal cord injury, cyclophosphamide induced inflammation, and feline interstitial cystitis, there is a significant increase in distension evoked ATP release (Sun and Chai 2002; Khera, Somogyi et al. 2004; Smith, Vemulakonda et al. 2005; Smith, Gangitano et al. 2008). In these cases, where tested, instillation of current therapeutic agents such as DMSO, heparin, and Botox, resulted in a significant reduction in distension evoked ATP. Botox was able to significantly attenuate distension evoked ATP release but had no effect on resting ATP release (Smith, Vemulakonda et al. 2005). This led to the proposal that there are two distinct mechanisms for ATP release, and only distension evoked release is mediated by exocytosis. This grants increased credibility to the prior work of Wang et al (2005) who proposed a role of ATP exocytosis in membrane trafficking and the maintenance of the urothelial barrier.

ATP release is also altered in human luminal samples from patients with OAB and has been proposed as a biomarker for OAB detection (Cheng, Mansfield et al. 2013; Silva-Ramos, Silva et al. 2013). Increased ATP release is observed in bladder strips from painful bladder, idiopathic detrusor overactivity, and neurogenic detrusor overactivity patients exposed to mechanical stretch (Kumar, Chapple et al. 2007; Kumar, Chapple et al. 2010). Observations such as these combined with the reductions in ATP release seen with Botox treatment, which is concurrent with reduced symptoms seen in human patients, afford a role for urothelially released ATP in bladder disease.

Regulation of non-neuronal ATP release

The control of urothelial ATP release is deemed essential to physiological bladder function and therefore must be carefully maintained. It has been shown that acetylcholine is also released in response to bladder distension (Yoshida, Inadome et al. 2006) and that acetylcholine binding to the urothelium is able to influence ATP release through activation of both nicotinic and muscarinic

receptors (Kullmann, Artim et al. 2008; Beckel and Birder 2012; Sui, Fry et al. 2014) via modulations in intracellular calcium signalling. This implies that there is a high level of coordination between cholinergic and purinergic mechanisms within the urothelium and the mechanism of action of currently applied muscarinic antagonists in LUT dysfunction could in fact be altering voiding reflexes through actions on non-neuronal ATP release. This could explain why antimuscarinic drugs currently used to manage overactive bladder show efficacy during the filling phase of the micturition cycle (Andersson and Yoshida 2003). ATP and activation of purinergic receptors has also been shown to induce acetylcholine release (Hanna-Mitchell, Beckel et al. 2007) as well as auto-feedback to influence ATP release itself (Sun and Chai 2005) which is blocked by the non-selective P2 antagonist suramin. The ATP metabolite adenosine, acting through P1 receptors inhibits further ATP release and this was proposed to be through inhibition of intracellular calcium liberation (Dunning-Davies, Fry et al. 2013). UTP has also been shown to significantly enhance ATP release via intracellular calcium pathways (Chopra, Gever et al. 2008; Sui, Fry et al. 2014) indicating that P2Y receptors are an essential component of the urothelial purinergic system, whilst αβMe-ATP, which preferentially activates $P2X_1$ and $P2X_3$ receptors had no effect on ATP release (Sui, Fry et al. 2014). These urothelial P2X receptors are assumed not to have an important role in the autocrine feedback to urothelial cells and this is partially verified by Valskovska et al (2001) who showed ATP release was unchanged in $P2X_3$ knockout mice. Also consistent with the proposed role of ATP as an auto feedback mechanism, is that fast desensitising channels such as $P2X_1$ and $P2X_3$ (North 2002) would not provide sustained increases in intracellular calcium during bladder filling in order to maintain mechanosensitivity and urothelial integrity.

Purinergic receptor expression in the bladder

The role of purinergic receptors and their agonists in bladder physiology in health and disease has been the focus of intense study for a number of years, and a number of important roles have been elucidated. Purinergic receptors have been identified within all major structures of the bladder including the detrusor smooth muscle (Andersson and Wein 2004), afferent nerves (Cockayne, Hamilton et al. 2000; Vlaskovska, Kasakov et al. 2001), interstitial cells (Wu, Sui et al. 2004; Sui, Wu et al. 2006; Cheng, Scigalla et al. 2011) and urothelium (Chopra, Gever et al. 2008; Shabir, Cross et al. 2013), of which the latter is of particular interest in this study.

The first study undertaken to identify purinergic receptors in the urothelium was performed using immunofluorescence in cats (Birder, Ruan et al. 2004). All seven P2X receptors and $P2Y_1$, $P2Y_2$ and

$P2Y_4$ were ubiquitous in both the basal and apical layers throughout the urothelium. A more rigorous approach to receptor identification was undertaken by chopra et al (2008). In rat urothelium, qPCR, western blotting, immunofluorescence and calcium imaging were undertaken to identify functional P2Y receptors. Calcium responses to UTP were found and attributed primarily to activation of $P2Y_2$ receptors. $P2Y_4$ was not seen with immunofluorescence in the bladder and a band was barely detectable in western blot studies. However, $P2Y_4$ mRNA was found to be present within urothelial cells (Chopra, Gever et al. 2008), and the $P2Y_{2/4}$ agonist UTP has since been shown to stimulate a rise in intracellular calcium in guinea pig urothelial cells (Sui, Fry et al. 2014). A comprehensive study of purinergic expression in human urothelial cells using RT-PCR has recently been published: there was minor expression of mRNA for $P2X_2$ but significant expression of $P2X_4$, $P2Y_1$, $P2Y_2$ and $P2Y_4$. A $P2Y_1$ selective agonist failed to evoke a rise in intracellular calcium in urothelial cells whereas both $P2Y_2$ and $P2Y_4$ agonists provoked robust calcium responses (Shabir, Cross et al. 2013).

Aims

The expression and function of purinergic receptors within the urothelium is of particular importance in understanding the role of ATP in bladder sensory signalling. As described above, an increase in urothelial ATP release and purinergic receptor expression has been shown to correlate with alterations in mechanosensation seen in patients with a number of overactive bladder syndromes. As the mechanisms of autocrine ATP signalling and ATP induced ATP release are deciphered, urothelial purinergic receptors have the potential to provide novel and specific targets for the treatment of overactive bladder, and because of their location in the periphery have the potential to treat symptoms with minimal adverse effects. Thus, an understanding of the receptors present in the urothelium, and their ability to mediate intracellular calcium concentrations is essential for mechanistic insight. Characterisation of purinergic receptor expression within urothelial cells of the mouse has yet to be performed and elucidation of these mechanisms will be crucial in deciphering the precise role of the urothelium in mechanotransduction pathways.

This chapter will focus on providing functional evidence for purinergic receptors within the urothelium.

3.2 EXPERIMENTAL PROTOCOLS

Isolation of primary mouse urothelial cells (PMUCs)

PMUCs were isolated as described in the main methods section. Briefly, following cervical dislocation, bladders were excised from the mouse and incubated with 2.5mg/ml Dispase in MEM for 2hrs at room temperature. Cells were scraped from the urothelium before being trypsinised (0.5% trypsin-EDTA (Invitrogen) at 37°C for 5-10 mins), and resuspended in fresh KSFM media. The cell suspension was counted and plated on collagen (IV) (Sigma- Aldrich (Poole,UK) coated coverslips and incubated overnight at 37°C in an atmosphere of 5% CO_2- 95% O_2 for use in both calcium imaging, qPCR and immunohistochemistry experiments the following day.

Calcium imaging

Cells attached to coverslips were incubated with 2µM Fura 2 acetoxymethyl ester (Fura2-AM) (Sigma- Aldrich (Poole,UK)) (30 min) at 37°C in the dark. Coverslips were then placed in a washing well containing only media at 37°C (30min) before being washed in HEPES solution at room temperature and then placed upon the recording equipment. Coverslips containing cells were then transferred to a perfusion chamber and mounted on an epifluorescent microscope and continually perfused with HEPES buffer at a rate of 1.5ml/min for 30 minutes. Cells for experiments carried out in normal calcium containing medium were exposed to the agonist for between 1-3 minutes via continual perfusion. If a second dose of agonist was to be applied, a 3 minute washout with HEPES was applied and then the agonist at the same concentration for a further 1-3 minutes. Finally the cells were stimulated with the calcium ionophore Ionomycin (5µM) (Sigma- Aldrich (Poole,UK)) as a positive control. All cells showing a positive response to Ionomycin (5 µM) (Sigma- Aldrich (Poole,UK)) exhibited robust responses to purinergic stimulation. Individual cells were easily discriminated based on fluorescent intensity under the microscope and for each experimental condition, at least 15 cells in at least three independent experiments were assayed. For experiments in nominal calcium solution, a period of two minutes was allowed prior to addition of the agonist to allow for the switch in solution. For experiments using antagonists, the antagonist was diluted from a stock solution and perfused continually in the HEPES for 30 minutes prior to and during the addition of an agonist. To ensure that cells were not desensitised to endogenous ATP released from basal cells in culture wells, in experiments examining the response of αβMe-ATP, coverslips were incubated with Apyrase

(Sigma- Aldrich (Poole,UK), an endonucleotidase able to rapidly metabolise ATP, for 1hr prior to being placed on the recording microscope.

Intracellular calcium was calculated as a ratio between the fluorescent signal at either 340/350 and 380nm (e.g. Rf 340/380) for responding cells. Results are also expressed as percentage of maximum Ionomycin response.

HEPES buffer for Urothelial cells (Composition in mM (NaCl 142, $NaHCO_3$ 5, HEPES 10, Glucose 16, KCL 2, $CaCl_2$ 2, $MgCl_2$ 1) as well as 0.1% BSA added to the dissolved solution.

HEPES buffer for Urothelial cells: Calcium free in mM (NaCl 142, $NaHCO_3$ 5, HEPES 10, Glucose 16, KCL 2, $MgCl_2$ 3) as well as 0.1% BSA added to the dissolved solution.

Drug	Action	Concentration
αβMe-ATP	P2X agonist	30μM
ATP	P2 agonist	10nM, 100nM, 1μM, 10μM, 100μM, 1mM
Apyrase	ATPase enzyme	1U-ml^{-1}
MRS2279	$P2Y_1$ antagonist	1μM
MRS2365	$P2Y_1$ agonist	100nM, 1μM, 10μM
MRS2578	$P2Y_6$ antagonist	1μM, 10μM
MRS2693	$P2Y_6$ agonist	10μM
NF449	P2X antagonist	1μM
PPADS	P2X antaogonist	30μM
UTP	$P2Y_{2/4}$ agonist	100μM, 10μM

Table 3.2.1 *A list of pharmacological tools used in this study.*

qPCR

Full details of qPCR experimental protocols are described in the major methods section.

mRNA was extracted from PMUCs 24hrs post isolation using Qiagen RNeasy minikit, converted to cDNA (superscript III Invitrogen) and frozen at -20°C prior to use in qPCR experiments.

An iQSYBR Green Super Mix (BioRad) was used to amplify the cDNA target genes in the qPCR reactions performed using a Research Rotor-Gene 6000 real time thermocycler (Corbett-Qiagen). The PCR reactions were made up to a total of 25μl containing 12.5μl of the iQSYBR Green master mix, 2μl of primers (1μl each of forward and reverse) and then a mix of cDNA and DEPC treated H_2O to

make up to 25µl. Cyclic conditions were set at: 95°C for 12 minutes as an initial hold stage followed by 40 cycles of 95°C for 30s, 59°C for 30s, 72°C for 30s, followed by a melt curve of 0.5°C increments every 30 seconds from 72-95°C.

Expression levels of each target gene were calculated relative to the housekeeping gene, β-actin, and represented here as a proportion of P2Y1 receptor expression.

Gene		Primer Sequence '5 – 3'	Product	Position	Tm°C	Chromosome
$P2X_1$	Forward	CAAGTATGCGGAGGACATGG	131bp	1362-	58.4	11
	Reverse	CACACTGAGTCAAGTCCGG		1493	57.8	
$P2X_2$	Forward	CCATCAGGTGAAGGACCAGC	118bp	1454-	60.4	5
	Reverse	GCTGGTCAAGAGTGTCCACC		1571	60.6	
$P2X_3$	Forward	CAGTGTTCACCAGTGACCAGGC	128bp	1086-	63.6	2
	Reverse	GCCTGTTGAAGGTTCTGCAGCC		1213	64.4	
$P2X_4$	Forward	CGTCTGTCACTCTAGAGACGG	202bp	1229-	59.3	5
	Reverse	GGTGCTGTTATGGACGTGTGG		431	61.5	
$P2X_5$	Forward	GTAGCCAGAGCTCTTGGCAGG	115bp	1494-	62.8	11
	Reverse	CTCAGAAGCCACATCCTGAGC		1608	60.8	
$P2X_6$	Forward	GACCTGCTGCTACTGTATGTGG	104bp	1120-	60.7	16
	Reverse	GGCTCGGTCTATGAACTGTTGG		1226	61	
$P2X_7$	Forward	CACATTCGCATGGTGGACCAGC	98bp	1213-	64.5	5
	Reverse	GACAGGTCGGAGAAGTCCATCTGG		1310	64.3	
$P2Y_1$	Forward	GTCTCAACAGCTGTGTGGACC	206bp	1598-	61	3
	Reverse	CTCAGGAGCTAGGATCTCGTGC		1803	62	
$P2Y_2$	Forward	CACGATGGACTTAGCTCAGAGG	207bp	1864-	60.2	7
	Reverse	CAGGAGGCAGAGATAACAGGC		2070	60.2	
$P2Y_4$	Forward	CAGCAGCTATGCAGAGGTAGC	194bp	553-	60.9	X
	Reverse	CCTCTGCCTGCAGTTAGTCC		746	60.1	
$P2Y_6$	Forward	GGCAACTGGTCAATTCATGC	150bp	1583-	58	7
	Reverse	CACATCCTGAGATGTCTAGC		1732	55	
$P2Y_{12}$	Forward	GTGTCAACACCACCTCAGCC	149bp	420-	61.1	3
	Reverse	CCTCATTGCCAAGCTGTTCG		568	59.8	
$P2Y_{13}$	Forward	GTTCCTCAAGATCATCATGCCG	210bp	436-	59.4	3
	Reverse	GTGTGACTGACCACCTGATGC		645	61.2	
$P2Y_{14}$	Forward	GTCACGAAGATACAGTGCATGG	131bp	792-	59.4	3

Table 3.2.2 *Primer sequence, product size, gene loci, and Tm for purinergic receptors investigated in this study.*

Immunohistochemistry

PMUCs on collagen coated coverslips were fixed with 1ml/well cold PFA (4%), washed, and permeabilised with sapponin (0.05% + FCS 2%) @ 4°C for 45 minutes. The primary antibody was prepared in saponin + FBS (1/200) and 50µl was applied to each coverslip before being left in the fridge overnight. Cells were washed and the secondary antibody (prepared in PBS + FBS 2% (1/200)) was applied (50µl) to each coverslip and left at room temperature in the dark for 2 hours. Coverslips were then mounted on slides using Vectashield® mounting media (containing Dapi) (Vector Laboratories, UK) before being left to dry in the dark and then imaged using a fluorescent microscope.

Primary antibody for urothelial cells was 1/50 Cytokeratin 7 Clone OV-TL 12/30 monoclonal mouse anti-cytokeratin 7 (DAKO) raised in mouse.

Secondary antibody used for urothelial cells was 1/1000 Alexa Fluor® 488 green goat anti-mouse (Invitrogen) raised in goat.

3.3 CHARACTERISATION OF PMUC RESPONSES TO ATP

Individual PMUCs within a single experiment responded to ATP (10µM) with variable increases in intracellular calcium expressed as a ratio of 340/380nm wavelength emissions (**fig 3.3.1**). Averaging of PMUC responses to ATP (10µM) show that intracellular calcium responses are characterised by two phases (**fig 3.3.2**). There was an initial rapid rise in intracellular calcium followed by a brief rapid decay, and a more sustained level of intracellular calcium, which was maintained until ATP was removed and a rapid return to baseline calcium levels was observed. This likely represents the contribution of a number of different purinergic receptors to the overall response.

The time course of the responses of PMUCs to increasing concentrations of ATP (10nM-1mM) can be seen in **fig 3.3.3** (N=6, n=360). PMUCs failed to respond to 10nM and 100nM ATP with an increase in intracellular calcium, but increasing doses (1µM, 10µM) elicited significant calcium fluorescence; reaching a peak at 100µM with no further increases upon application of 1mM ATP. The concentration response relationship to ATP can be seen in **fig 3.3.4**, where intracellular calcium reaches a peak following 100µM ATP with a pEC50 of 3.49±0.77µM (N=6, n =360).

Confocal images were used to show immunohistochemical staining of PMUCs (**fig 3.3.5**). Confluent urothelial cells expressed cytokeratin 7 (green) in the plasma membrane, with DAPI labelling of the nucleus. Light microscope images taken immediately after (A) and 30 minutes following (B) urothelial cell placement onto collagen IV coated coverslips (**fig 3.3.6**) reveal that PMUCs migrate towards each other to form a confluent monolayer typical of epithelial cells. The hexagonal morphology of the urothelial cells can also be seen following monolayer formation.

An initial application of ATP (10µM) to PMUCs caused a significant rise in intracellular calcium (0.23±0.03, p≤0.0001, N=3, n=67, paired t-test) above baseline, reaching a peak after 15.3±1.4seconds before exhibiting a slow decline and a return to baseline following agonist washout (**fig 3.3.7**). A subsequent dose of ATP (10µM) to the same cells, following a 3 minute washout with krebs solution also initiated a significant rise in intracellular calcium (0.15±0.02). There was a significant decline in the maximal response to 10µM ATP between the first and second applications (p≤0.0001, N=3, n=67, Wilcoxon matched-pairs signed rank test). The percentage decrease in calcium fluorescence (**fig 3.3.8**) of the second ATP response is equal to 30.5±2.8% of the first. There was also a significant change in the kinetics of PMUC responses to ATP (10µM) seen upon repeat applications (**fig 3.3.9**). Initial applications of 10µM ATP reached maximal calcium fluorescence after

15.3±1.4 seconds with a significant increase in the time taken to reach peak calcium fluorescence upon a subsequent activation (19.50±2.3s) ($p \leq 0.05$, N=3, n=67, paired t-test).

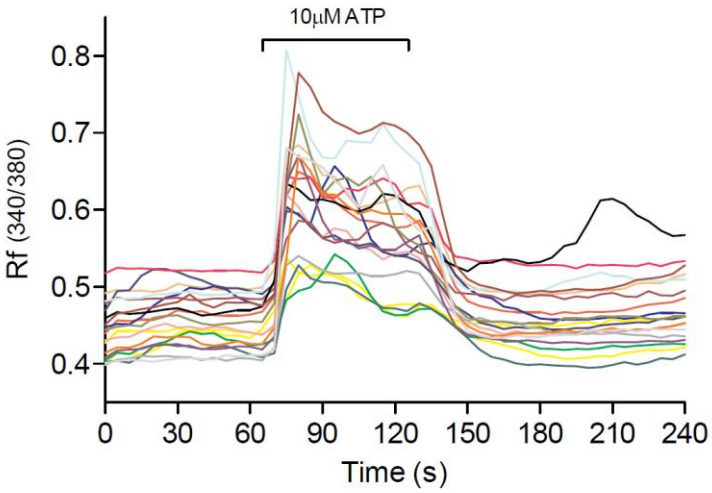

Fig 3.3.1 *Experimental trace showing the variability in individual PMUC intracellular calcium responses to ATP (10μM) from a single experiment (n=23).*

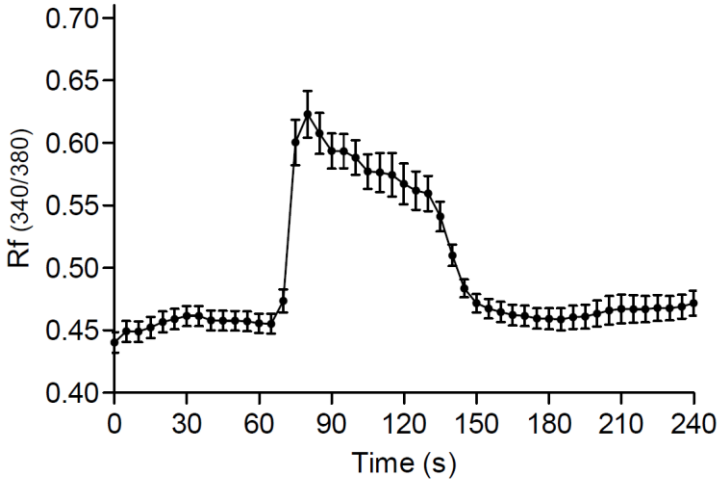

Fig 3.3.2 *Mean±(SEM) change in intracellular calcium levels of PMUCs to ATP (10μM) from a single experiment (n=23).*

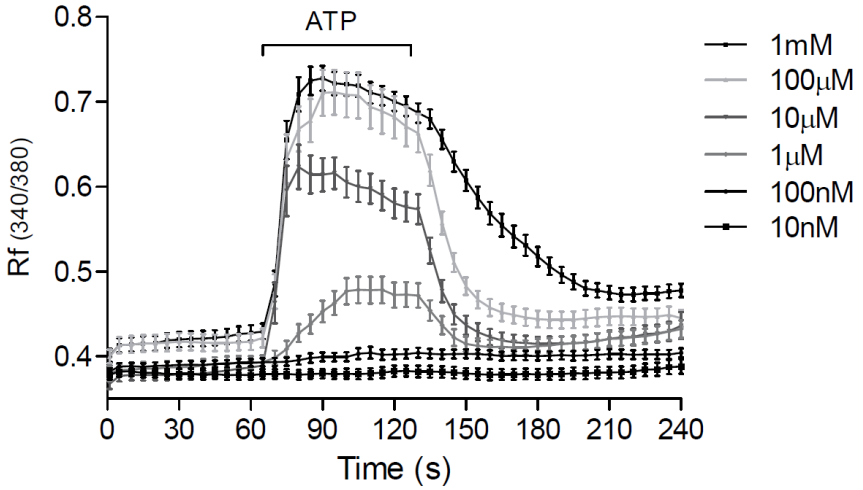

Fig 3.3.3 *Mean±(SEM) time course response of PMUCs to increasing concentrations of ATP (10nM-1mM), (N=6, n=360).*

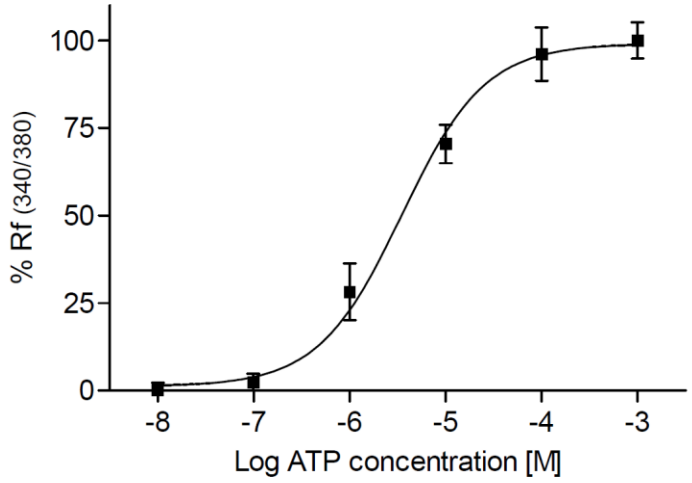

Fig 3.3.4 *Mean±(SEM) concentration response curve of PMUC intracellular calcium to ATP (10nM-1mM) (N=6, n=360). Data normalised to % of maximal response.*

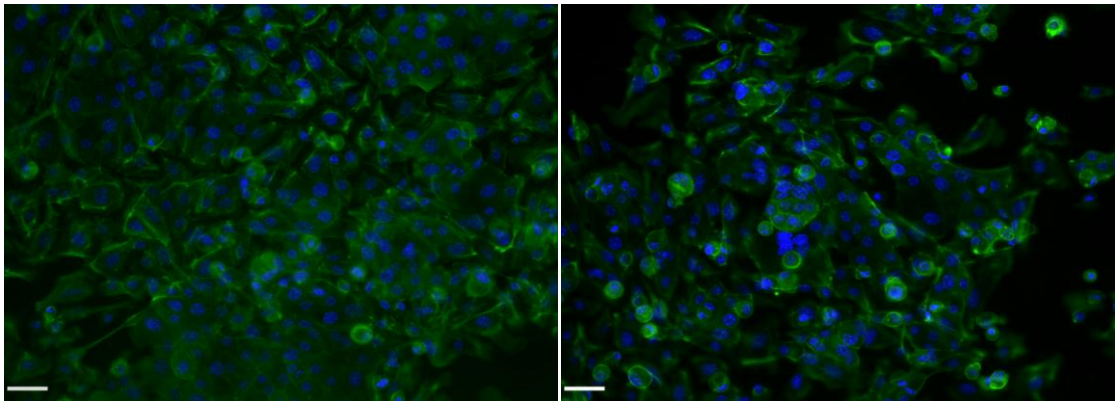

Fig 3.3.5 *Immunohistochemistry of cultured PMUCs. Cytokeratin 7 (green) is stained in the plasma membrane of urothelial cells. Blue, DAPI, stains the nucleus. Scale bars, 20µm.*

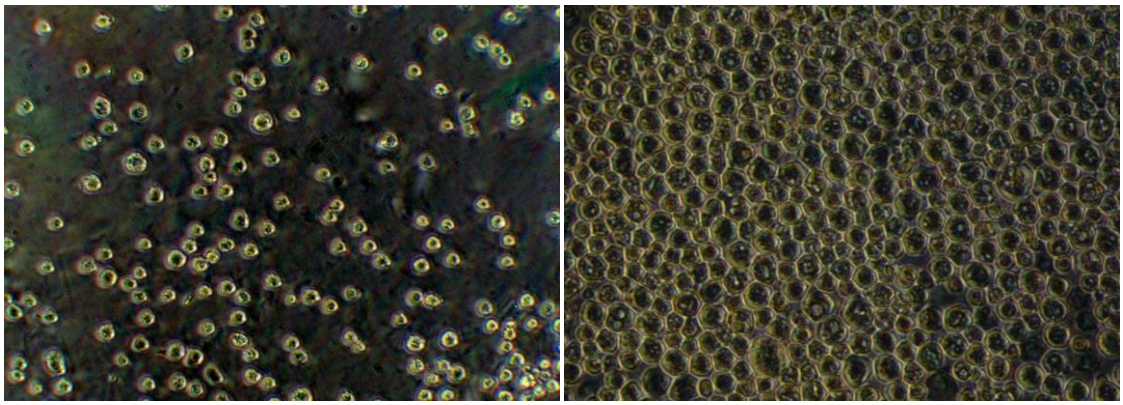

Fig 3.3.6 *Light microscope images of PMUCs immediately following isolation and plating on collagen IV coated coverslips (A) and after 30min (B).*

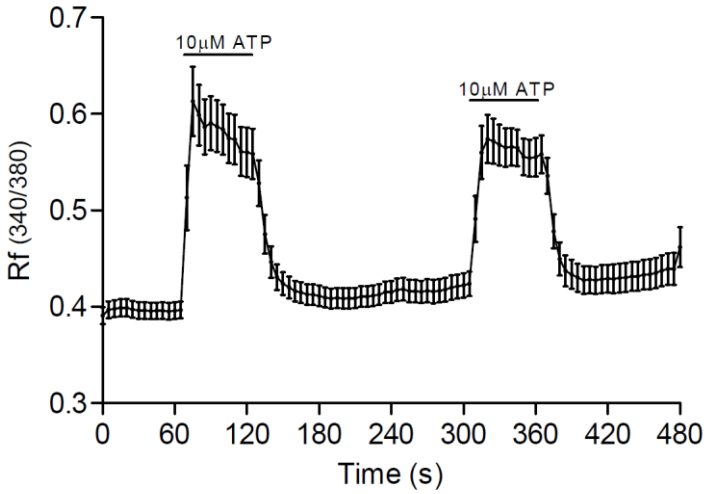

Fig 3.3.7 *Mean±(SEM) time course of intracellular calcium response of PMUCs to repeat applications of ATP (10µM) with washout, (N=3, n=67).*

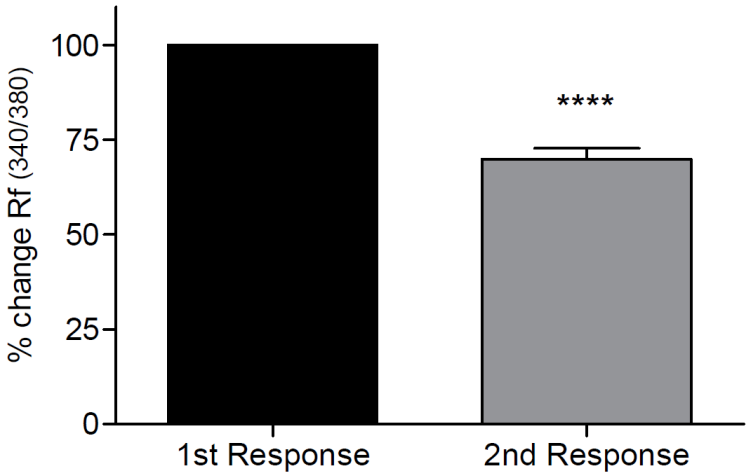

Fig 3.3.8 *Mean±(SEM) percentage change in PMUC intracellular calcium in response to repeat applications of ATP (10µM) with washout (****p≤0.0001, N=3, n=67).*

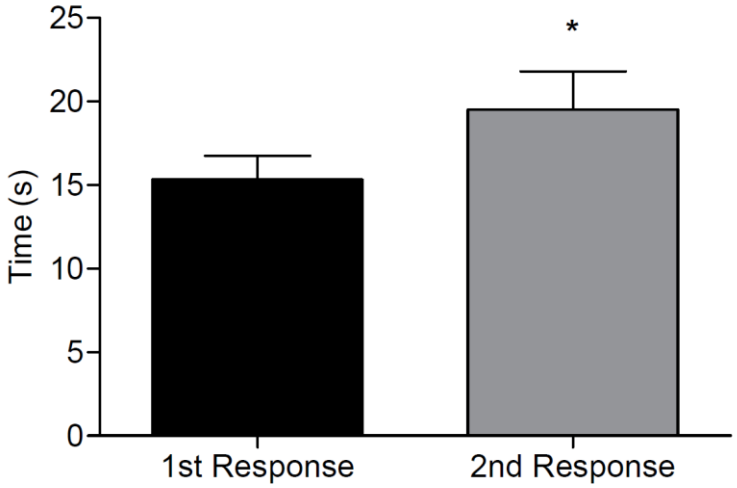

Fig 3.3.9 *Mean±(SEM) time taken for PMUC to reach peak intracellular calcium in response to repeat applications of ATP (10µM) with washout (*p≤0.05, N=3, n=67).*

3.4 THE ROLE OF EXTRACELLULAR CALCIUM IN PMUC RESPONSES TO ATP

Application of 10µM ATP to PMUCs in calcium free HEPES (**fig 3.4.1**) evoked a significant increase (0.16±0.03) in intracellular calcium above baseline (p≤0.0001, N=3, n=62, paired t-test). In the absence of calcium, the characteristic two phase change in intracellular calcium seen in control experiments (**fig 3.4.2**) had disappeared, instead replaced by a response which, although still rapidly activated, did not exhibit an initial sharp peak, and instead showed a slow decline in response until eventually returning to baseline following washout of agonist. A subsequent dose of 10µM ATP also caused a significant rise in intracellular calcium (0.16±0.03) above baseline (p≤0.0001, N=3, n=62, paired t-test). However, there was no change in the maximal intracellular calcium response to ATP between the first and second applications in calcium free HEPES solution (p≥0.05, N=3, n=62, paired t-test).

Maximum intracellular calcium response above baseline to duplicate applications of ATP (10µM) in PMUCs in the presence or absence of extracellular calcium are summarised in **fig 3.4.2.** The peak intracellular calcium response to ATP (10µM) was significantly decreased following a prior application of ATP at an equal concentration in normal HEPES solution. There was no significant change in maximum intracellular calcium responses with duplicate application of 10µM ATP when extracellular calcium was removed. There was also no significant difference between the second ATP (10µM) response in normal HEPES and those responses seen in calcium free HEPES (p≥0.05, N=3, n=62, one-way ANOVA, Bonferroni multiple comparisons post-hoc test). A significant increase in the time taken (15.3±1.4s Vs 23.3±1.9s) to reach peak intracellular calcium in response to ATP (10µM) was observed in the absence of extracellular calcium (p⩽0.01, N=3, n=67, 62, un-paired t test (***fig 3.4.3***))

Concentration related responses of PMUCs to ATP (10nM-1mM) were observed in the presence and absence of extracellular calcium, and responses to maximal doses of ATP (100µM, 1mM) (**fig 3.4.4**) were not significantly different (p=0.4 N=6, unpaired t-test). 10nM and 100nM ATP also failed to initiate a rise in intracellular calcium in calcium free HEPES. Changes in responses to ATP (10nM-1mM) by removal of extracellular calcium are represented by a significant shift to the right on the concentration response curve; pEC50 3.49±1.34 Vs 9.5±1.5µM (p=0.013, N=6, unpaired t-test, (**fig 3.4.5**)).

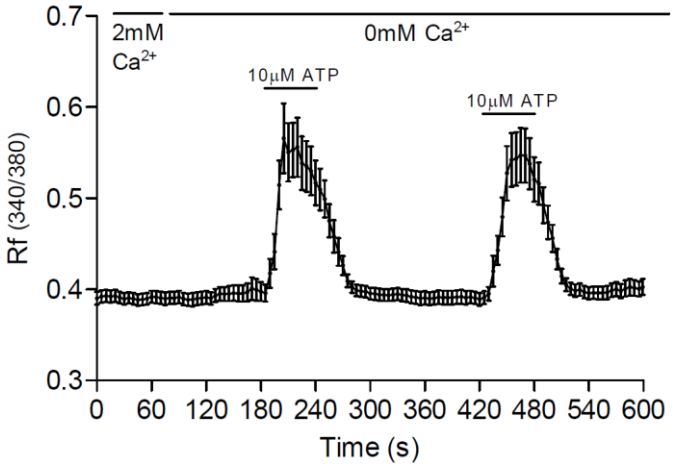

Fig 3.4.1 *Mean±(SEM) time course of intracellular calcium response of PMUCs to repeat applications of ATP (10µM) in 0mM Ca^{2+} HEPES solution with washout, (N=3, n=62).*

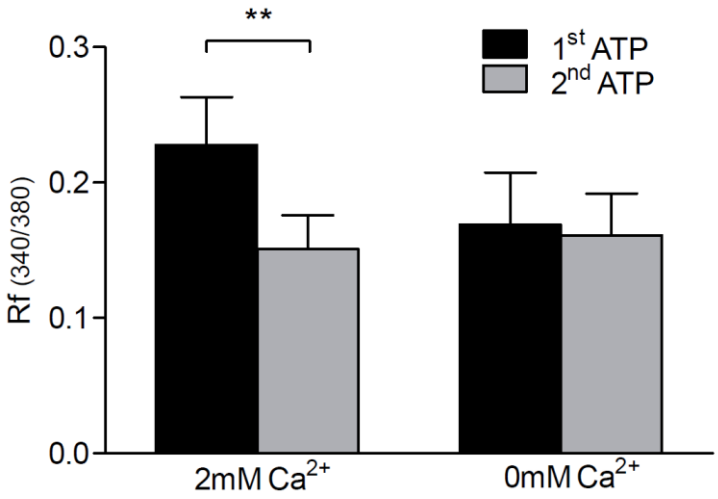

Fig 3.4.2 *Mean±(SEM) peak intracellular calcium increase above baseline for repeated doses of ATP (10µM) on PMUCs in normal and calcium free HEPES solution, (***p≤0.01, N=3, n=67, 62).

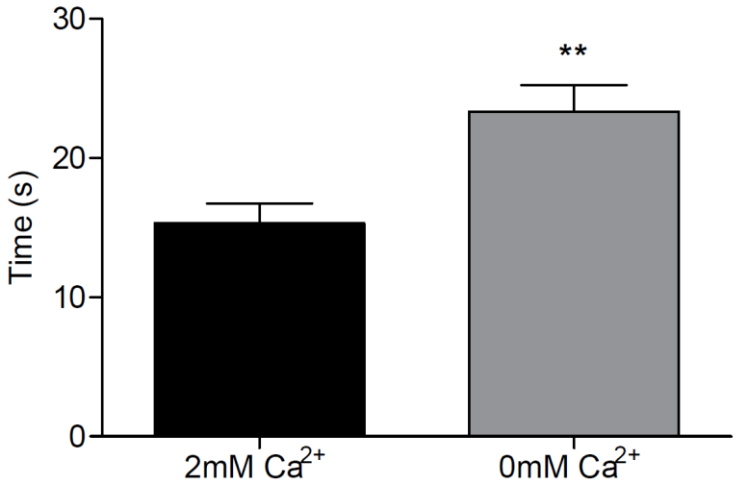

Fig 3.4.3 *Mean±(SEM) time taken for PMUC to reach peak intracellular calcium in response to ATP (10μM) in the presence and absence of extracellular calcium (**p≤0.01, N=3, n=67, 62)*

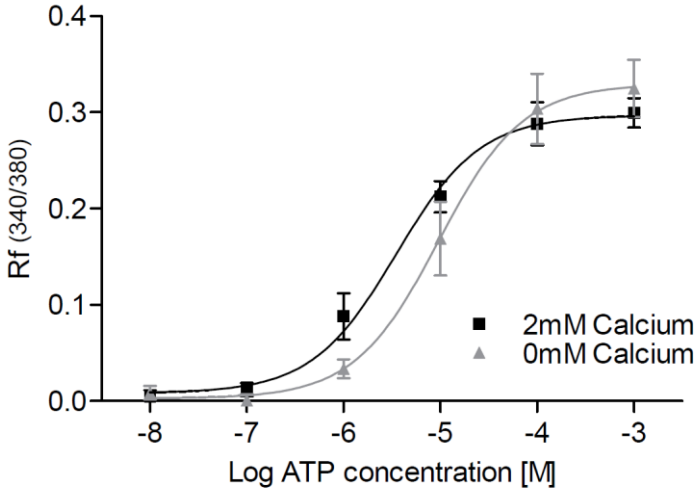

Fig 3.4.4 *Mean±(SEM) concentration response curve of PMUC intracellular calcium to ATP (10nM-1mM) (N=6, n=360, 314) in normal and calcium free HEPES solution.*

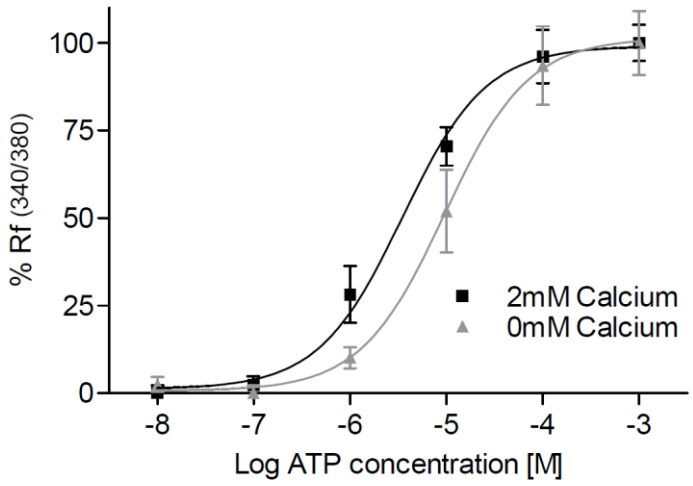

Fig 3.4.5 *Mean±(SEM) concentration response curve of PMUC intracellular calcium to ATP (10nM-1mM) in normal and calcium free HEPES solution. Data normalised to a percentage of maximal response.*

3.5 THE CONTRIBUTION OF P2X IN PMUC RESPONSES TO ATP

The relative expression of purinergic receptor mRNA in PMUCs (**fig 3.5.1**) revealed $P2X_2$ receptor mRNA was expressed in the highest amounts within the urothelium (N=3), whilst mRNA for all other purinergic receptors except $P2X_7$ was detected within the urothelium. Of the G-protein coupled P2Y receptors, mRNA for the $P2Y_1$ and $P2Y_2$ receptors was highest.

RT-qPCR provides insight into the mRNA levels of the purinergic receptors within the urothelium, the next section of this chapter will provide evidence towards functional receptors for a number of the above receptors.

Application of the P2X agonist αβMe-ATP (30μM) to PMUCs stimulated a significant ($p \leq 0.0001$, N=3, n=72, paired t-test) increase in intracellular calcium (**fig 3.5.2**). The calcium response to αβMe-ATP (30μM) was characterised by a sharp increase to a peak of 0.31±0.05 above baseline calcium levels, reaching a peak following 9±2.8seconds after agonist application, followed by a steady decline in response to 45% of the maximum during the 60 second application of the agonist before returning back to baseline following washout.

Prior incubation of the $P2X_1$ receptor antagonist NF449 (1μM) before ATP (10μM) application did not block ATP (10μM) induced intracellular calcium increases (**fig 3.5.3**) and did not significantly attenuate maximal responses to ATP (10μM) (**fig 3.5.6**) ($p \geq 0.05$, N=3, n=68, One-way ANOVA, Dunnett's multiple comparisons post-hoc test). However, NF449 (1μM) significantly alters the intracellular calcium response profile of ATP (10μM) which exhibits a significantly delayed onset of action (**fig 3.5.7**). The latency of the response to ATP (10μM) following prior incubation of NF449 (1μM) is shown in **fig 3.5.7**. The time taken to reach peak intracellular calcium levels in response to ATP (10μM) was increased from 15.5±1.4s in control experiments to 29.2±2.1s following NF449 (1μM) ($p \leq 0.001$, N=3, n=68, one way ANOVA, Dunnett's multiple comparisons post-hoc test).

Similarly, ATP (10μM) was able to induce an increase in intracellular calcium with prior incubation of the P2X antagonist PPADS (30μM) (**fig 3.5.4**). As with NF449 (1μM), PPADS (30μM) did not significantly alter maximal fluorescent responses to ATP (10μM) (**fig 3.5.6**) ($p \geq 0.05$, N=3, n=63, one-way ANOVA, Dunnett's multiple comparisons post hoc-test) but showed significant effects on the time taken to reach a maximal response to ATP (10μM) (**fig 3.5.7**). Single cell responses forming an experimental trace can be seen in **fig 3.5.5** and show an altered calcium response profile when compared to control (**fig 3.3.2**). The time taken to reach peak calcium fluorescence in response to

ATP (10μM) was increased from 15.5±1.4s in control to 45.9±2.1s with PPADS (30μM) ($p \leq 0.001$, N=3, n=68). There was also a significant difference between the time to peak response of ATP (10μM) between NF449 (1μM) and PPADS (30μM) treatments ($p \leq 0.001$, N=3, n=68, 63, One-way ANOVA, Dunnett's multiple comparisons post hoc-test) (**fig 3.5.7**).

These data show that PMUCs cultured over 24hrs are able to respond to P2X stimulation by αβMe-ATP (30μM) and that the P2X component of ATP (10μM) induced calcium influx can be modified by P2X specific antagonists NF449 (1μM) and PPADS (30μM). However, there still remains a large proportion of the intracellular calcium response to extracellular ATP and it is proposed that P2Y receptors are responsible for these actions.

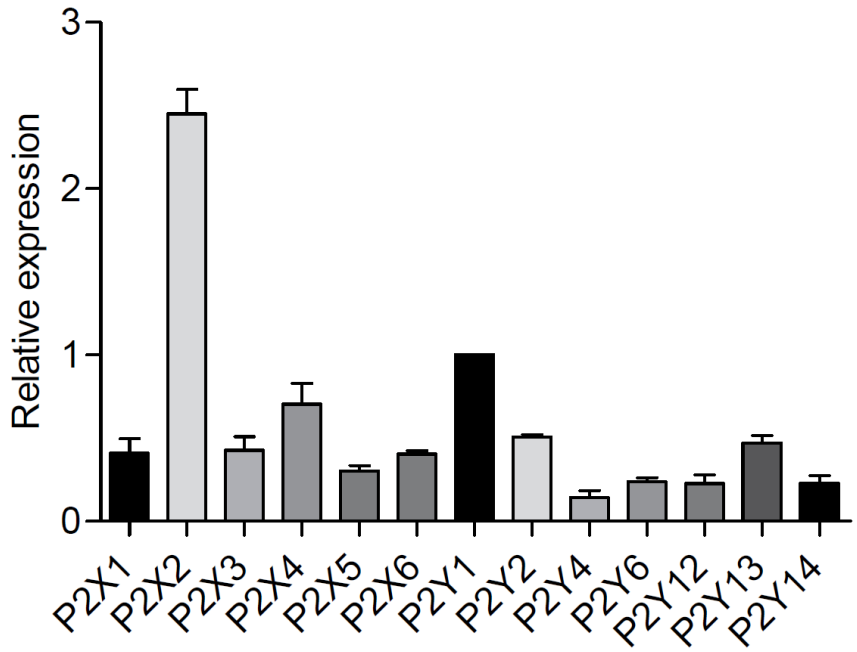

Fig 3.5.1 *RT q-PCR of purinergic receptors within cultured mouse urothelial cells. Data represented as an expression relative to that for the P2Y$_1$ receptor (N=3).*

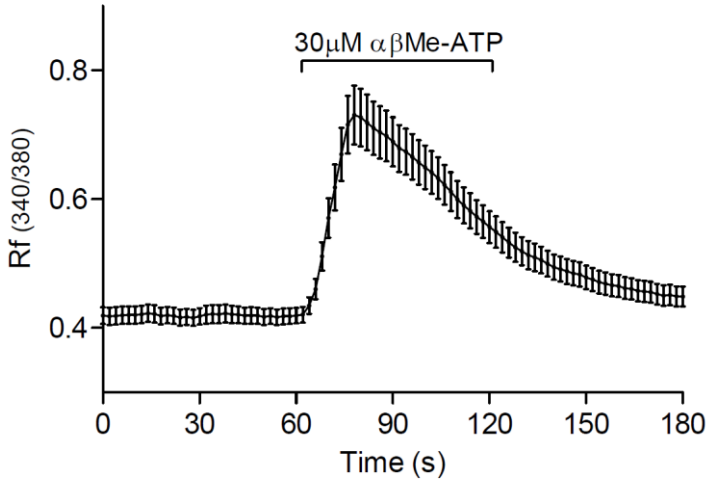

Fig 3.5.2 *Mean±(SEM) change in intracellular calcium levels of PMUCs to αβMe-ATP (30μM)* (N=3, n=72).

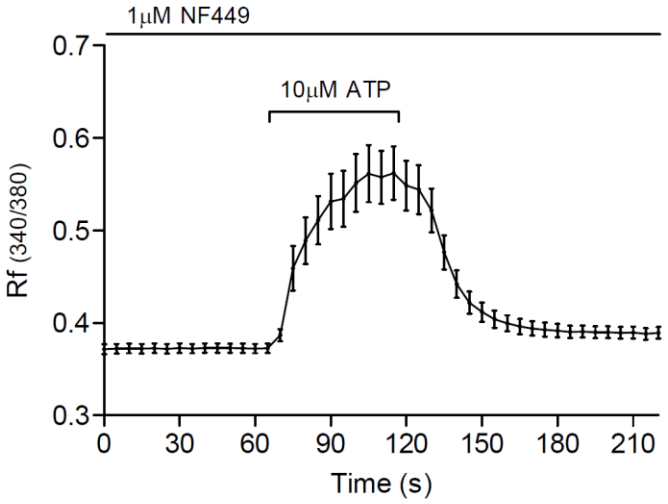

Fig 3.5.3 *Mean±(SEM) change in intracellular calcium levels of PMUCs to ATP (10μM) in the presence of NF449 (1μM) (*N=3, n=68).

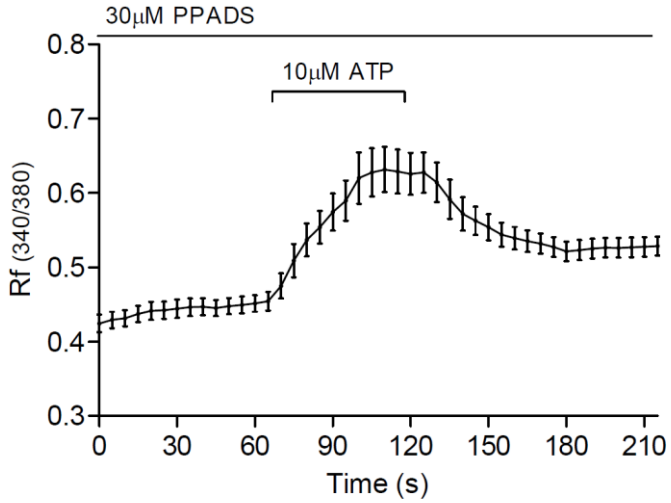

Fig 3.5.4 *Mean±(SEM) change in intracellular calcium levels of PMUCs to ATP (10μM) in the presence of PPADS (30μM) (N=3, n=63).*

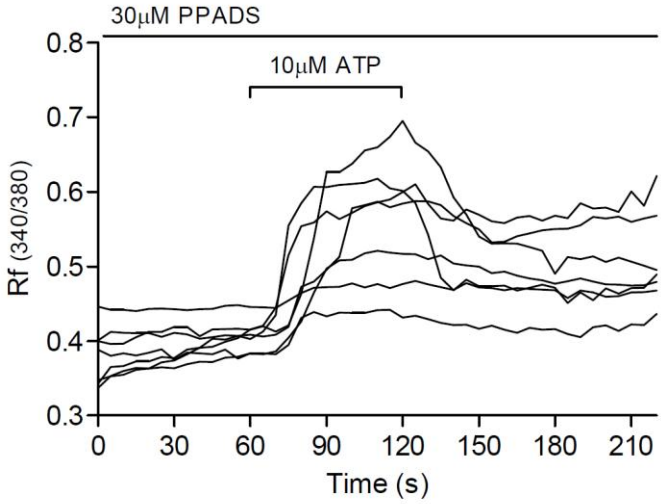

Fig 3.5.5 *Experimental trace showing individual PMUC intracellular calcium responses to ATP (10μM) in the presence of PPADS (30μM).*

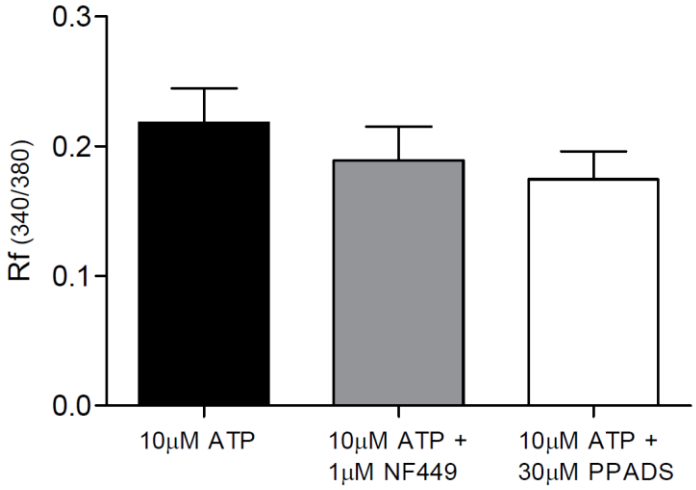

Fig 3.5.6 *Mean±(SEM) peak intracellular calcium response of PMUCs above baseline to ATP (10μM) in the absence and presence of NF449 (1μM, n=68) or PPADS (30μM, n=63).*

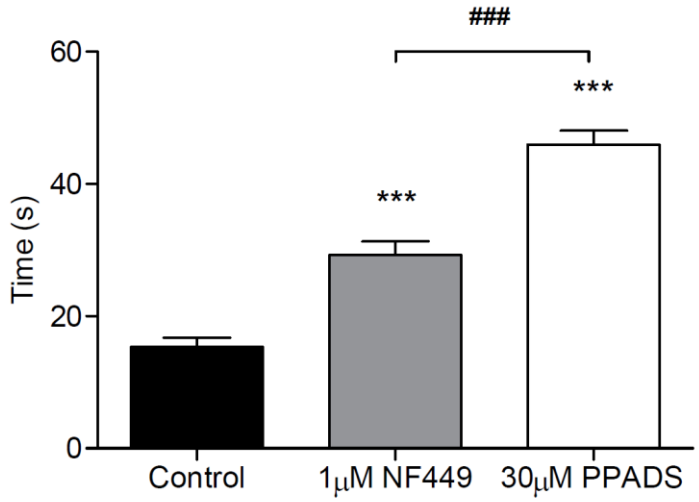

Fig 3.5.7 *Mean±(SEM) time taken for PMUCs to reach peak intracellular calcium in response to ATP (10μM) in the presence and absence of NF449 (1μM) (***p≤0.001 Vs control, n=68), and PPADS (30μM) (***p≤0.001 Vs control, n=63).*

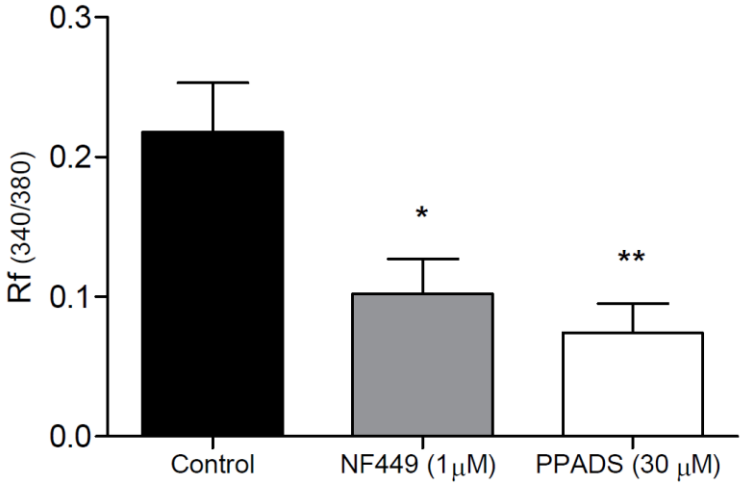

Fig 3.5.8 *Mean±(SEM) peak intracellular calcium response of PMUCs to ATP (10μM) in the presence and absence of NF449 (1μM) (*p≤0.05 Vs control, n=68) and PPADS (30μM) (**p≤0.01 Vs control, n=63) 15s after ATP application.*

3.6 THE CONTRIBUTION OF P2Y RECEPTORS

The specific P2Y receptor agonist UTP (10μM, 100μM) caused a concentration dependent increase in intracellular calcium (**fig 3.6.1**) (N=5, 123, 98). The increase in intracellular calcium initiated by UTP (100μM) reaches 63.2% of the maximum to an equivalent concentration of ATP (N=5, n=121). The response profile of UTP (10μM) activation can be seen in the time course response of **fig 3.6.2**. UTP (10μM) initiated a rise in intracellular calcium which was maintained for the duration of agonist application and returned to baseline only after the agonist was removed from the bath.

The time course response to duplicate applications of UTP (10μM), which can be seen in **fig 3.6.2**, shows repeat applications of UTP (10μM) did not show any obvious changes in response profile. As well as a similar response profile, the maximum intracellular calcium response to UTP (10μM) was not significantly different between first and second responses (**fig 3.6.4**), ($p \geq 0.05$, N=3, n=73, Wilcoxon matched-pairs signed rank test).

The $P2Y_6$ agonist MRS2693 (10μM) caused a small, but significant ($p \leq 0.001$, N=3, n=61), paired t-test, **fig 3.6.6**) rise in PMUC intracellular calcium. The effect of $P2Y_6$ activation on intracellular calcium was dose dependently antagonised (**fig 3.6.5**) by a $P2Y_6$ specific antagonist MRS2578: 1μM (7.8±0.3 Vs 4.0±0.3% ionomycin, $p \leq 0.001$, N=3, n=77), and 10μM (7.8±0.3 Vs 2.0±0.3% ionomycin, $p \leq 0.001$, N=3, n=58, one-way ANOVA, Dunnett's multiple comparisons post-hoc test).

The $P2Y_6$ receptor antagonist MRS2578 (10μM) also significantly inhibits UTP (10μM) evoked increases in intracellular calcium (**fig 3.6.7**) (12.9±0.5 Vs 9.4±1.2% ionomycin, $p \leq 0.05$, N=4, n=65, unpaired t-test).

The $P2Y_1$ receptor agonist MRS2365 caused a dose dependent (100nM 1μM 10μM) increase in PMUC intracellular calcium (**fig 3.6.8**) (N=4, n=80, 79, 91). The time course of the response of MRS2365 (1μM) can be seen in **fig 3.6.9**. As with UTP (10μM) and the $P2Y_6$ agonist MRS2693 (10μM), the intracellular calcium response to MRS2365 did not show desensitisation in the continued presence of the agonist, and responses are reduced to baseline only following agonist wash out.

The $P2Y_1$ receptor antagonist MRS2279 (1μM) was able to significantly attenuate MRS2365 (10μM) induced calcium fluorescence (19.5±0.8 Vs 5.3% max ionomycin, $p \leq 0.0001$, N=3, n=58, **fig 3.6.10**), and MRS2365 (100nM) (7.3±0.4 Vs 2.4±0.2% ionomycin, $p \leq 0.0001$, (N=3, n=74) one-way ANOVA, Bonferroni multiple comparisons post-hoc test)

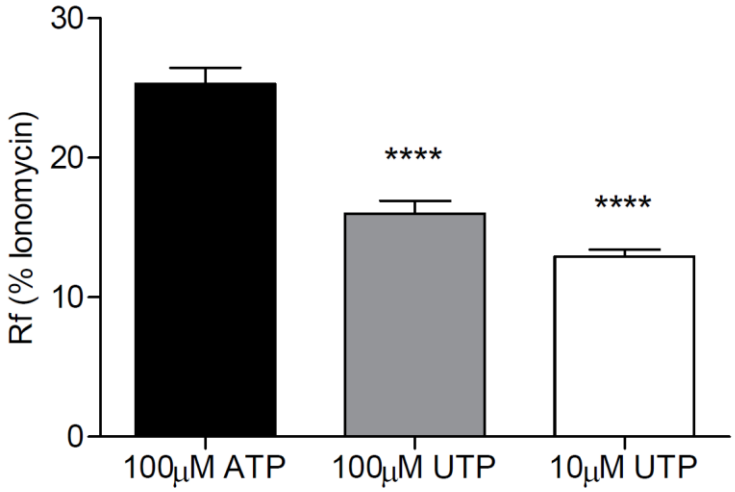

Fig 3.6.1 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of UTP (100μM N=5, n=*98*, 10μM n=*123*) and ATP (100μM N=5, n=121), (****p≤0.0001 Vs control).*

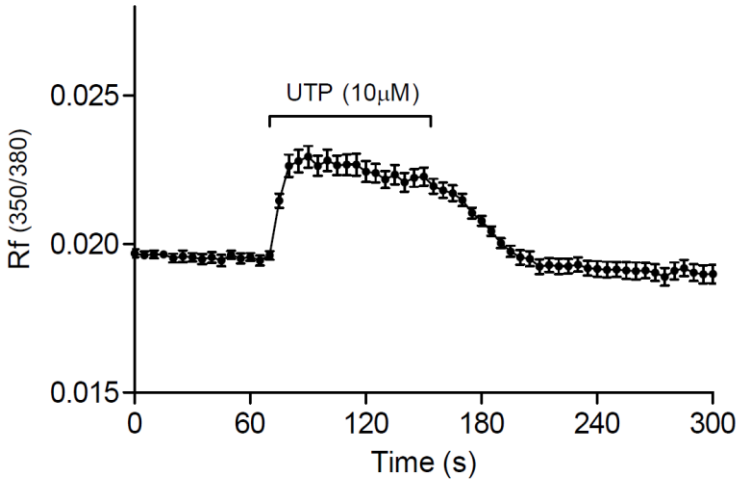

Fig 3.6.2 *Mean±(SEM) change in intracellular calcium levels of PMUCs to UTP (10μM) (N=5, n=123).*

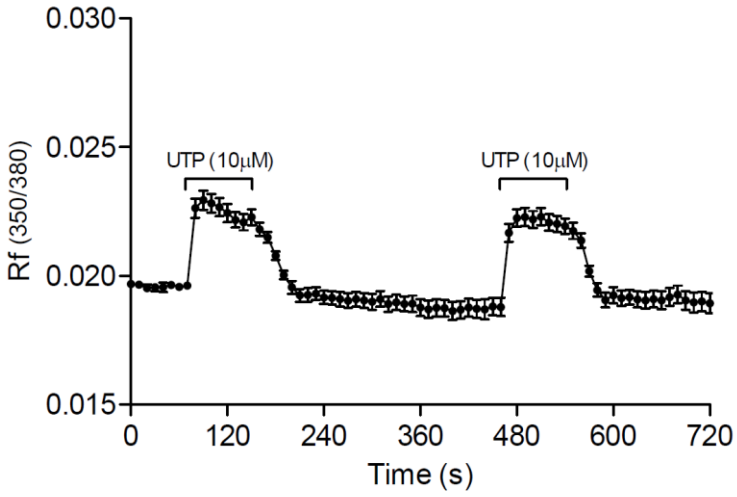

Fig 3.6.3 *Mean±(SEM) time course of intracellular calcium response of PMUCs to repeat applications of UTP (10µM) with washout, (N=3, n=73).*

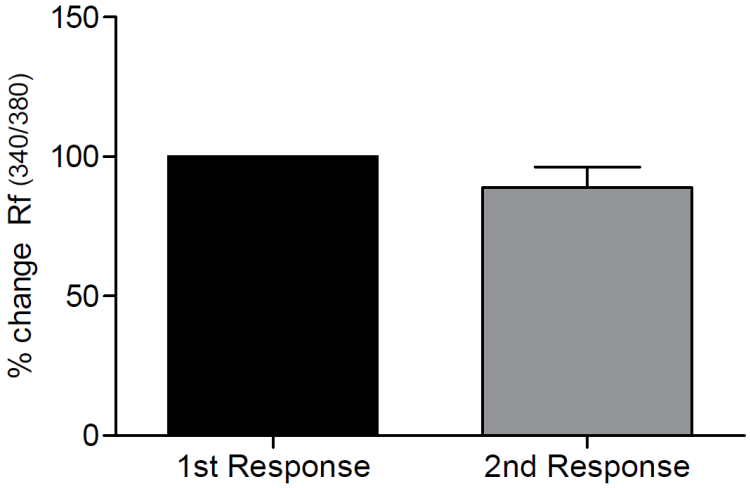

Fig 3.6.4 *Mean±(SEM) percentage change in PMUC intracellular calcium in response to repeat applications of UTP (10µM) with washout (N=3, n=73).*

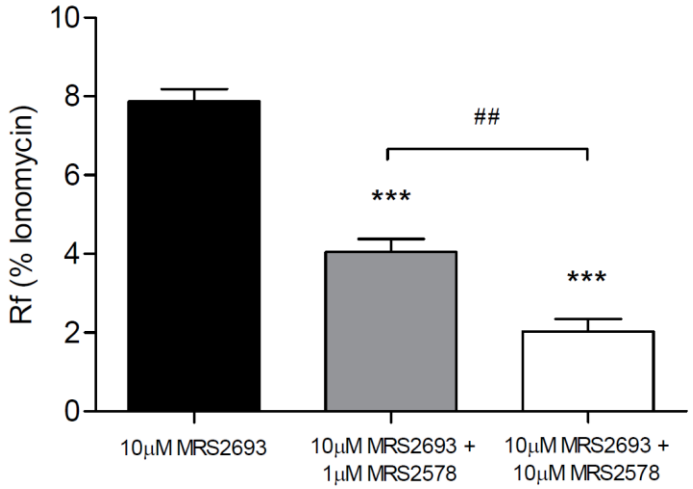

Fig 3.6.5 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of $P2Y_6$ agonist MRS2693 (10μM) in the absence and presence of $P2Y_6$ antagonist MRS2578 1μM (***p≤0.001, N=3, n=77) and 10μM (***p≤0.001 Vs control, N=3, n=58)*

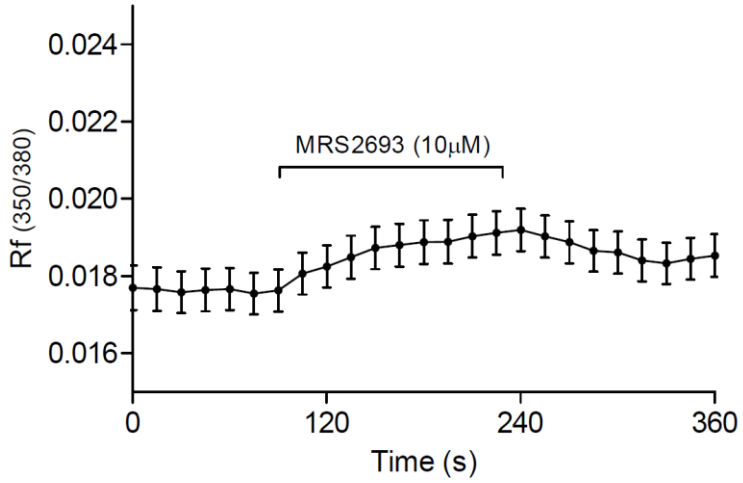

Fig 3.6.6 *Mean±(SEM) change in intracellular calcium levels of PMUCs to $P2Y_6$ agonist MRS2693 (10μM, N=3, n=61)*

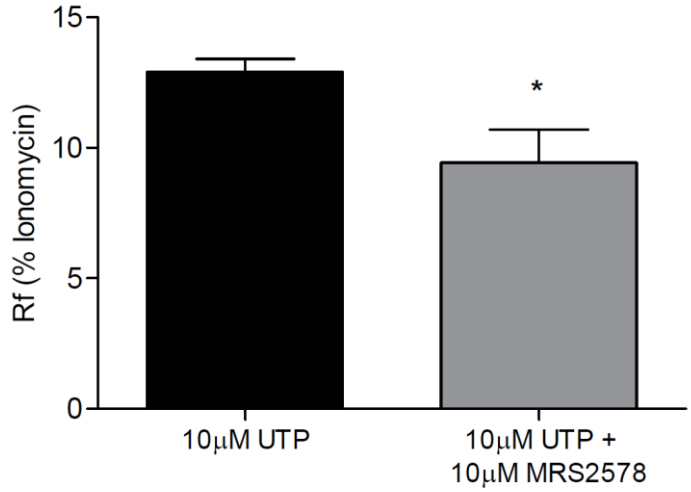

Fig 3.6.7 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of UTP (10μM) in the absence (n=*123*) and presence of the P2Y$_6$ antagonist MRS2578 (10μM) (*p≤0.05, N=4, n=65).

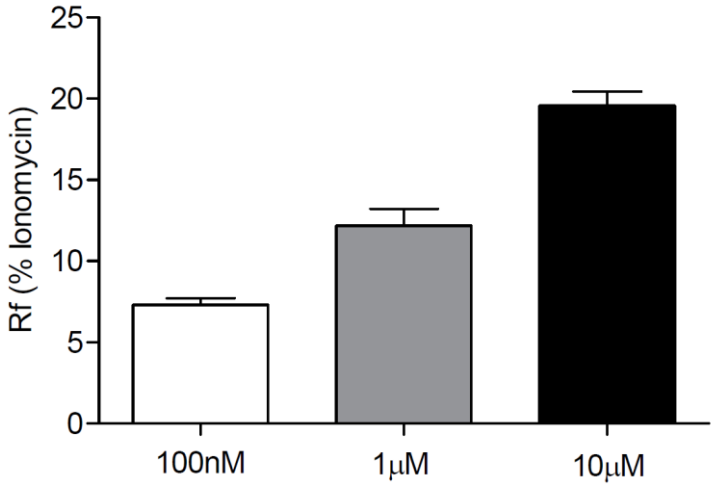

Fig 3.6.8 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of the P2Y$_1$ agonist MRS2365 (N=4, 100nM (n=80), 1μM (n=79), 10μM (n=91).*

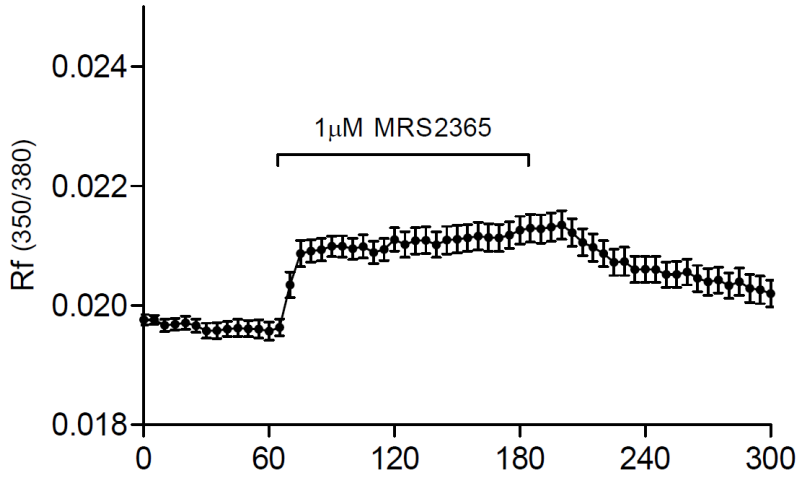

Fig 3.6.9 *Mean±(SEM) change in intracellular calcium levels of PMUCs to $P2Y_1$ agonist MRS2365 (1μM, N=4, n=79).*

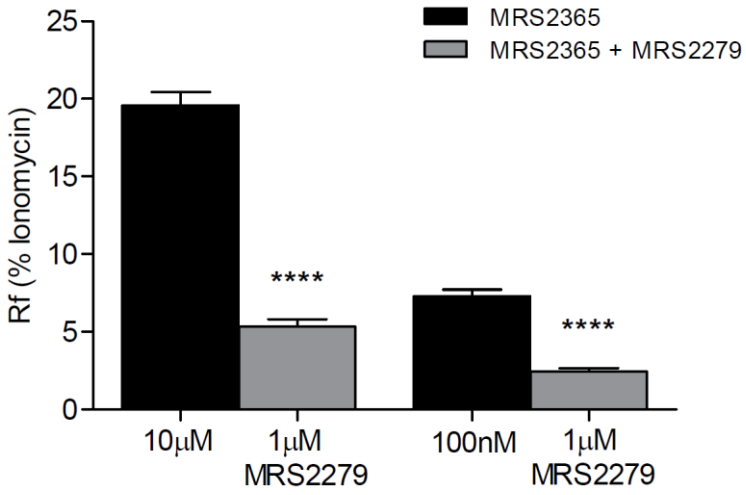

Fig 3.6.10 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of $P2Y_1$ agonist MRS2365 (10μM, n=91, 100nM, n=80) in the absence and presence of the $P2Y_1$ antagonist MRS2279 (1μM) (*****p≤0.0001 Vs agonist alone, N=3, n=58, n=74)

	mRNA Expression	Ca2+ Response		mRNA Expression	Ca2+ Response
P2X1	**	***	**P2Y1**	***	***
P2X2	****	^^^	**P2Y2**	**	***
P2X3	**	***	**P2Y4**	*	***
P2X4	***	^^^	**P2Y6**	*	*
P2X5	*	-	**P2Y12**	*	-
P2X6	*	-	**P2Y13**	**	-
P2X7	0	-	**P2Y14**	*	-

Fig 3.6.11 *Tabular summary of the experimental data for this chapter.* ******* *= (Functional response/gene expression),* ***^*** *= (Speculative functional response)* ***0*** *= (No functional response/gene expression),* ***-*** *= (not tested).*

3.7 DISCUSSION

The data presented in this study reveal the functional presence of purinergic receptors within the urothelium of the mouse bladder. Using intracellular calcium imaging, it has been demonstrated that PMUCs respond to both P2X and P2Y receptor agonists. Specific agonists and antagonists reveal the important role of P2Y receptors, specifically $P2Y_1$, $P2Y_2$ and $P2Y_4$ in mediating urothelial responses to endogenous agonists. The results are supported by mRNA expression of purinergic receptors.

PMUCs were confirmed to be epithelial in origin through staining with cytokeratin 7 and were also observed to migrate towards each other following their plating onto collagen coated coverslips where they formed a monolayer exhibiting typical hexagonal morphology. The ability of urothelial cells to migrate towards each other points to the release of a multitude of factors that enable them to communicate with each other, as well as a most probable functional coordination that requires their confluence.

ATP induced calcium response

A number of previous studies have shown functional purinergic receptors and responses to ATP on the urothelium (Chopra, Gever et al. 2008; Everaerts, Vriens et al. 2010; Shabir, Cross et al. 2013; Sui, Fry et al. 2014). In the present study, urothelial responses to ATP were consistent with previous observations of ATP induced increases in intracellular calcium. The ATP response was characterised by an immediate sharp increase followed by a slight decrease and sustained intracellular levels of calcium in the presence of the agonist. It was shown that PMUCs respond to exogenous ATP with a concentration dependent increase in maximum intracellular calcium. Based on the characteristics of the response to ATP, a number of things can be inferred. The initial rapid increase in intracellular calcium suggests that ionotropic P2X channels contribute to this component of the response through influx of extracellular calcium (North 2002). Following a slight decrease, a more sustained level of intracellular calcium develops for the remainder of ATP application. Again this indicates a number of possible interactions. Ionotropic P2X receptors show marked desensitisation following rapid activation (North 2002), and this would be characteristic of the initial desensitisation phase. Secondly, there is a sustained level of intracellular calcium with continued ATP application which is more characteristic of metabotropic P2Y mediated intracellular calcium liberation through G-protein coupling of IP_3 (von Kügelgen 2006).

The response profile to ATP provides significant clues to the functional capabilities of urothelial cells and this discussion will focus on determining the role of P2X and P2Y receptors in initiating a rise in intracellular calcium and the potential consequences in relation to bladder function.

It was found that with repeated applications of sub-maximal doses of ATP, with a short washout period, there was a significant reduction in the maximal intracellular calcium response. As intracellular calcium levels had returned to baseline prior to a second dose of agonist, it is likely that the decrease in response is due to desensitisation of urothelial purinergic receptors responsible for calcium influx. This desensitisation significantly reduces the ability of cells to increase intracellular calcium levels in response to subsequent ATP and also alters the kinetics of the response, with a second dose of ATP taking a significantly longer time to reach peak intracellular calcium levels. However, as these results demonstrate, a large proportion of the response remains, suggesting more than one population of receptors is responsible for the differential components of the intracellular calcium response. As ATP is known to bind to multiple purinergic receptors, the effects observed can be most likely attributed to responses of fast desensitising ionotropic P2X receptors (North 2002) and metabotropic P2Y receptors, as both of these receptor subtypes have previously been identified within the urothelium of a number of other species (Birder, Ruan et al. 2004; Tempest, Dixon et al. 2004; Chopra, Gever et al. 2008; Sui, Fry et al. 2014).

To further test the contribution of P2Y receptors in mediating intracellular calcium responses to ATP, experiments were performed in the absence of extracellular calcium in an attempt to remove the ionotropic P2X component of calcium influx in urothelial cells. PMUCs responded to ATP with a significant increase in intracellular calcium even in the absence of extracellular calcium. This provides further evidence that there is a large proportion of the urothelial response to ATP which is mediated by the liberation of calcium from intracellular stores rather than through membrane bound calcium channels, a characteristic of P2Y receptor function. It is also of importance to note that with repeated applications of ATP in the absence of extracellular calcium there was no reduction in peak response or obvious change in response profile. The ability of urothelial cells to respond to continuous or repeated stimuli with an increase in intracellular calcium, the known stimulus for distension evoked ATP release from the urothelium (Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011), is essential to the proposed physiological role of urothelial signalling during bladder distension. This leads to the hypothesis that activation of P2Y receptors are an essential component in autocrine signalling of urothelial cells.

Interestingly, the maximal intracellular calcium increase above baseline in experiments without extracellular calcium is almost identical to the maximal response seen following receptor

desensitisation in normal HEPES. This further supports the suggestion that the receptors responsible for the response to ATP in the absence of extracellular calcium are most likely the same as those responsible for the non-desensitised component of ATP induced intracellular calcium rise in normal calcium containing solution.

Further evidence to support multiple populations of purinergic receptors within the mouse urothelium is provided by the shift to the right of the concentration-response curve seen in the absence of extracellular calcium and a significant increase in the pEC50 for ATP. Interestingly, the attenuation in intracellular calcium seen within the pEC50 range of ATP concentrations in calcium free HEPES are not seen with higher ATP concentrations. This could imply that at maximal concentrations of ATP, ionotropic purinergic activation does not contribute significantly to the levels of intracellular calcium within the urothelium. It is hard to determine the importance of these results however, as the levels of ATP recorded from bladders at rest and during distension are most commonly in the nano and pico-molar range (Vlaskovska, Kasakov et al. 2001; Collins, Daly et al. 2013; Hanna-Mitchell, Wolf-Johnston et al. 2013) which, as it is diluted into a much larger volume of either urine or saline is in no way indicative of the level of ATP released at the level of the urothelial cell membrane. Studies into levels of ATP release however, do provide vital information on the changes occurring between basal and stretch of the urothelium and in response to exogenous stimuli.

The importance of exogenous ATP mediated intracellular calcium levels within the urothelium is implicated by the role of calcium in mediating stretch evoked ATP release (Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011; Dunning-Davies, Fry et al. 2013), the ability of ATP to induce ATP release (Sun and Chai 2005), and the multitude of interactions that ATP has been proposed to mediate in the sub-urothelium relating to mechanosensitivity. Thus, a mechanism by which the actions of ATP and its metabolites are able to mediate further ATP release could be an additional mechanism contributing to the enhancement in reflex bladder activity observed in a number of bladder disorders.

Purinergic Receptor Expression

RT-qPCR of PMUCs found expression of mRNA for all P2X and P2Y receptors tested except the $P2X_7$ subtype. $P2X_1$, $P2X_2$ and $P2X_3$ receptors have been shown to be expressed in human urothelium (Liu, Xu et al. 2013) whilst $P2X_7$ is selectively expressed on cells of hematopoietic lineage as well as glial cells, Schwann cells and astrocytes (Skaper, Debetto et al. 2010). Previous studies investigating $P2X_7$

receptor expression in urothelial cells have reported mixed results. Shabir et al (2013) found no mRNA expression of $P2X_7$ in human urothelium, whilst an immunohistochemistry study of cat urothelium (Birder, Ruan et al. 2004) revealed significant $P2X_7$ staining throughout the basal and apical layers of the urothelium. These differences could be attributed to the species used; however, as has been suggested recently, there is a large amount of non-specific adsorption of antibodies in the urothelium (Yu and Hill 2011) which was exemplified in experiments on TRPV1 expression (Everaerts, Sepúlveda et al. 2009) and thus evidence based on immunohistochemistry alone should be considered with care. Another possible explanations for this discrepancy could be the presence of stromal cells within the urothelium, however, no changes in $P2X_7$ immunoreactivity were observed in a model of interstitial cystitis (Birder, Ruan et al. 2004), which has previously been shown to induce stromal cell proliferation (Vera, Iczkowski et al. 2008), and thus this seems unlikely.

The most significantly expressed purinergic receptors from PMUCs were $P2X_2$, $P2X_4$, $P2Y_1$ and $P2Y_2$, as well as lesser expression of other purinergic receptor subtypes including $P2Y_6$. This is consistent with the results of Chopra et al (2008) who, although only looking at $P2Y_2$ and $P2Y_4$ receptors within the urothelium, identified higher expression of $P2Y_2$. The $P2Y_6$ receptor has previously been localised to the urothelium of rat bladder using immunofluorescence confocal microscopy (Timóteo, Carneiro et al. 2014). In the human urothelium, $P2Y_1$ and $P2Y_2$ mRNA are expressed (Liu, Xu et al. 2013; Shabir, Cross et al. 2013), yet interestingly, in cultured cells, expression of $P2Y_4$ receptor mRNA was upregulated (Shabir, Cross et al. 2013). As the cells used in the present study were only in culture for 24hrs post-isolation, and details of days in culture are not provided in other studies, it is assumed that the results in this study represent those of a normal urothelial phenotype. Phenotype expression has previously been explored in mouse urothelial tissue versus 24hr cultured cells, and no differences were observed (Everaerts, Vriens et al. 2010). Another considerable difference between the current study and that of Shabir et al (2013) is the precise quantification that is afforded to the qPCR techniques of this study as opposed to a qualitative analysis from an agarose gel.

Functional P2X receptors

The expression of $P2X_2$ and $P2X_4$ is consistent with an ionotropic mediated component of the ATP response and this was further investigated. αβMe-ATP shows significant specificity for P2X receptor subtypes, particularly $P2X_1$ and $P2X_3$. Despite comparatively low mRNA expression levels of these receptors, αβMe-ATP was able to induce a significant increase in intracellular calcium. It was

observed that the response of PMUCs to αβMe-ATP incurred significant desensitisation during agonist application due to the fast activation and deactivation kinetics of this subtype of purinergic receptor (North 2002). The actions of αβMe-ATP in the current study are in contrast to those of others who found no functional response to αβMe-ATP, however, to obtain the results seen in these experiments, it was essential to incubate cells with the ATP'ase apyrase prior to αβMe-ATP application to ensure the receptors were not desensitised, a methodology not previously employed (Shabir, Cross et al. 2013; Sui, Fry et al. 2014). The emergence of these responses however, provokes an important discussion. Due to the continual release of ATP from the urothelium, not only during stretch but also whilst at rest, what is the physiological relevance of these responses? Given the availability of ectonucleotidases able to rapidly breakdown ATP in the vicinity of the urothelium and sub-urothelium (Yu, Robson et al. 2011), there is the possibility that desensitisation of P2X receptors is more of a feature when bath application of ATP swamps the ability of ectonucleotidases to terminate their action.

PMUC responses to ATP were also examined in the presence of the P2X receptor antagonists NF449 and PPADS. It was found that neither NF449 nor PPADS significantly attenuated maximal responses to ATP, however, the time taken to reach maximal fluorescence was significantly increased and thus a large difference between maximal response to ATP fifteen seconds after application was observed. Other attempts to antagonise the P2X component of calcium influx have been attempted, PPADS has been shown to attenuate ATP responses but was also found to inhibit UTP responses (Chopra, Gever et al. 2008), and ATP responses in calcium free external solution (Shabir, Cross et al. 2013) suggesting that PPADS is able to impose non-P2X specific actions. NF449 is highly selective for the $P2X_1$ receptor and these experiments provide evidence for a role of the $P2X_1$ receptor in mediating urothelial calcium responses to ATP.

The role of intracellular calcium in distension evoked ATP release has been confirmed (Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011), and ATP has been shown to stimulate further ATP release (Sun and Chai 2005). Nonetheless, the functional importance of the initial fast component of ATP induced calcium influx is not known. The ATP induced ATP release observed by sun et al (2005) was inhibited by the non-selective purinergic antagonist suramin, and ATP release from the urothelium is not affected by αβMe-ATP (Sui, Fry et al. 2014) in mucosal strips or following knockout of the $P2X_3$ receptor (Vlaskovska, Kasakov et al. 2001). This conforms to the idea that ionotropic receptors would be tonically desensitised in normal urothelial tissues and their functional relevance in mediating distension evoked ATP release is limited. In contrast, it has been shown that intravesical αβMe-ATP is able to stimulate afferent nerve activity (Rong, Spyer et al. 2002)

and was proposed by the author to act either via urothelial cells or directly on afferent nerve terminals. The dose of αβMe-ATP used in this study was high, at 1mM, and conclusions about the receptors to which it binds are thus not possible. Application of the agonist in this study was also accompanied by changes in intravesical pressure, so responses may have been secondary to mechanical changes (Rong, Spyer et al. 2002). Intravesical application of 10mM ATP has also been shown to induce bladder overactivity via an afferent mechanism (Pandita and Andersson 2002) suggesting that ATP is able to stimulate the micturition reflex. Again however, the behavioural effects seen by Pandita et al (2002) could either be through direct activation of sub-urothelial afferent nerves or mediated through activation of urothelial cells. With application of agonists into the lumen of the bladder, it is difficult to draw conclusions either way. Further than this, however, due to the dose of agonist used, conclusions about the receptors responsible are still unclear. Based on the experiments performed above it would be my hypothesis that the actions of ATP on the urothelium are mediated via P2Y receptors.

Functional P2Y receptors

The observation of ATP-induced rises in intracellular calcium that are independent of extracellular calcium would implicate metabotropic P2Y receptors in urothelial ATP responses. The presence of E-NTPDase ectonucleotides in the urothelial layer (Yu, Robson et al. 2011), which have the ability to breakdown ATP to the $P2Y_1$ agonist ADP, and results showing that UTP is able to stimulate release of ATP (Chopra, Gever et al. 2008; Sui, Fry et al. 2014) have all provided further credibility to the theory that P2Y receptors have an essential role in urothelial function and ATP release. Indeed, as shown in **table 1.7.1**, the ATP metabolite ADP shows greatest affinity for $P2Y_1$ receptors and $P2Y_2$ and $P2Y_4$ receptors bind both ATP and UTP with equal potency. The $P2Y_6$ receptor, however, although showing some affinity for ATP, is preferentially activated by uridine molecules UDP and UTP.

As previously described, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA is expressed in PMUCs with $P2Y_1$ and $P2Y_2$ receptor expression highest. The presence of functional P2Y receptors was therefore further investigated in the current study. UTP, an agonist at a number of P2Y receptors which shows greatest affinity for $P2Y_2$ and $P2Y_4$ was able to stimulate a concentration-dependent increase in intracellular calcium. Urothelial responses to UTP showed no desensitisation following repeat applications. The ability of UTP to stimulate an increase in urothelial intracellular calcium has been observed in other species. In rat cultured urothelial cells, UTP has been shown to stimulate intracellular calcium rises (Sui, Fry et al. 2014) via phospholipase C-linked mechanisms which were

unaffected by extracellular calcium but significantly attenuated by store depletion (Chopra, Gever et al. 2008). UTP-stimulated calcium increases in this study were accompanied by significant and reproducible ATP release which has been replicated in guinea pig urothelial strips (Sui, Fry et al. 2014). In human ureter-derived urothelial cells, functional expression of both $P2Y_2$ and $P2Y_4$ receptors has been observed (Shabir, Cross et al. 2013).

Functional responses to highly selective $P2Y_1$ and $P2Y_6$ receptor agonists in PMUCs were observed in the present study, and these responses were found to be attenuated by their respective antagonists. $P2Y_1$ mediated responses were robust and showed concentration dependency. $P2Y_6$ mediated responses were the smallest of all P2Y responses investigated, however, the $P2Y_6$ receptor antagonist was also found to significantly attenuate a portion of the calcium response to UTP. The $P2Y_1$ receptor has been previously found not to be functionally responsive in ureter-derived urothelial cells, although the concentration of agonist used in this study, at 1nM, was significantly lower (Shabir, Cross et al. 2013). Despite this, application of a specific $P2Y_1$ antagonist to urothelial cells in the current experiments was able to almost abolish the response to the $P2Y_1$ receptor agonist and was therefore considered specific to the $P2Y_1$ receptor. This is the only other study that has previously examined the role of $P2Y_1$ in urothelial cells and the difference in functional receptors could reflect a difference in physiological function as the cells in the study by Shabir et al (2013) were derived from the ureters. Ureters have a significant amount of anatomical homology to the bladder but do not require the same functions. The ureters are responsible for the transport of urine from the kidneys to the bladder, and as such, are only exposed to transient light distension, much like the peristaltic waves present in the gastrointestinal tract. Thus, there is less physiological evidence for a role by which sustained distension induced release of ATP and the maintenance of intracellular calcium concentrations is warranted. Indeed, ureter distension occurs during the passage of kidney stones which are extremely painful and perhaps the phenotype of these receptors more closely resembles a role of ATP in nociception.

The function of the $P2Y_6$ receptor has not been previously investigated in isolated urothelial cells and thus no comparisons to other studies can be drawn. Activation of the $P2Y_6$ receptor upon the urothelium however, has been investigated to some extent. Instillation of a stable UDP analogue selective for $P2Y_6$ receptors into the bladder of anesthetised rats increases voiding frequency without affecting the amplitude or duration of cystometrically measured bladder contractions. These effects were blocked by a $P2Y_6$ antagonist. The UDP analogue also increased ATP in the urine (Timóteo, Carneiro et al. 2014).

The functional significance of the results presented here have yet to be determined, however, the presence of functional purinergic receptors, particularly P2Y is hypothesised to play an essential role in micturition. Both ATP and UTP have been shown to induce intracellular calcium increases in urothelial cells and consequential ATP release. The sustained increase in intracellular calcium observed with UTP and P2Y activation is likely to be of physiological relevance, maybe more so than a desensitising response of P2X receptors to ATP, as during mechanosensation of the bladder, sensation must be maintained throughout the filling phase up to the threshold for micturition and if ATP is a key factor, its release must be continual. ATP and UTP have equal potency at rodent $P2Y_2$ and $P2Y_4$ receptors and it could be hypothesised that the rise in intracellular calcium with ATP in calcium free media is a result of $P2Y_2$ and $P2Y_4$ receptor activation, with a possible small contributing role of $P2Y_6$ activation. Unfortunately, there are no selective $P2Y_2$ and $P2Y_4$ antagonists and this theory cannot be fully investigated. The $P2Y_1$ receptor is preferentially activated by ADP and ATP, and could also play a significant role in extracellular calcium independent ATP mediated urothelial responses. Intracellular calcium is a major contributing pathway for ATP release (Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011) and increases in intracellular calcium by ATP and UTP are accompanied by the release of non-neuronal ATP from the urothelium which is not influenced by specific P2X agonists (Sui, Fry et al. 2014) or $P2X_3$ receptor knockout (Vlaskovska, Kasakov et al. 2001) but is blocked by the non-selective purinergic antagonist suramin (Sun and Chai 2005), further supporting a role for urothelial P2Y receptors in mediating this process. The role of urothelial ATP has been explored in the bladder and it has been found to be a mediator of detrusor smooth muscle contraction, interstitial cell function and afferent nerve excitability and thus the ability to mediate these responses through antagonism of P2Y receptors upon the urothelium is a particularly attractive pharmacological target. Indeed, $P2Y_1$ and $P2Y_2$ receptors have been shown to be upregulated in the urothelium of interstitial cystitis patients (Liu, Xu et al. 2013) and this is accompanied by an increase in urothelial ATP release.

A secondary hypothesis, supported by evidence in other organs, but not yet explored in the bladder, is that UTP, along with ATP is released from the urothelium during mechanical stretch. Mechanically released UTP has the potential to act on the ubiquitous P2Y receptors on urothelial and underlying cells to potentiate an increase in intracellular calcium and further ATP and UTP release as well as to influence mechanosensation. Murine airway epithelial cells exhibit propagation of mechanically induced calcium waves which are dependent on mechanically induced UTP release (Homolya, Steinberg et al. 2000) and mechanical stimulation of astroma cells has also been shown to release UTP (Lazarowski, Homolya et al. 1997). Development of an assay for the quantification of UTP has lead to the discovery of UTP release in a number of cells including primary and cultured airway

epithelial cells in both resting and mechanically stimulated conditions (Lazarowski and Harden 1999) which were increased up to 20 fold using a medium displacement method. Although this was not the classical hypotonic stretch that is often used to characterise urothelial cell responses, it supports the hypothesis that epithelial cells are capable of releasing UTP and the mechanism for the downstream modulation of the micturition reflex is apparent.

Conclusions

These results have shown for the first time that functional P2Y receptors are present on the mouse urothelium and their activation results in significant increases in intracellular calcium. The importance of these findings is not yet clear, but the control of urothelial intracellular calcium levels is a necessary factor in ATP release, and ATP release is an essential component in the control of micturition within the bladder. In addition, the results in this study using primary mouse urothelial cells show a number of similarities with the human urothelium and support the use of mice as a significant tool in the continued search to determine urothelial cell function.

CHAPTER 4: THE ROLE OF TRPV1 IN P2X MEDIATED BLADDER AFFERENT SIGNALLING

4.1 INTRODUCTION

The TRPV1 receptor is a member of the TRPV subfamily of TRP ion channels permeable to monovalent and divalent cations. TRPV1 is activated by vanilloid compounds (such as capsaicin and resiniferatoxin [RTX]), noxious temperature (>44˚C), and acid (<pH 6.0) and thus has an essential role in the integration of noxious stimuli. The threshold for TRPV1 activation is reduced by inflammatory mediators such as NGF, bradykinin (Chuang, Prescott et al. 2001), and ATP (Tominaga, Wada et al. 2001).

Where is TRPV1 found within the bladder?

Many immunohistochemical studies have demonstrated the presence of TRPV1 in DRG neurons (Hwang, Min Oh et al. 2005) supplying the bladder and on nerve fibers throughout the lower urinary tract. There is significant immunoreactivity of the TRPV1 receptor on non-myelinated C-fibers (Tominaga, Caterina et al. 1998; Yiangou, Facer et al. 2001; Avelino, Cruz et al. 2002), but also larger diameter A-fibers (Yiangou, Facer et al. 2001; Ost, Roskams et al. 2002) present in muscular and mucosal plexuses of the bladder. As well as the documented presence of TRPV1 within the peripheral endings of sensory afferents, there is evidence of TRPV1 immunoreactivity within the interstitial cells (Ost, Roskams et al. 2002), smooth muscle cells (Birder, Kanai et al. 2001; Ost, Roskams et al. 2002), and more controversially, the urothelium. TRPV1 expression has been described in human urothelial cell cultures (Lazzeri, Vannucchi et al. 2004; Apostolidis, Brady et al. 2005), but not detected by others (Ost, Roskams et al. 2002). Expression has also been described in rat urothelial cells (Birder, Kanai et al. 2001; Kullmann, Shah et al. 2009) but not in the mouse (Yamada, Ugawa et al. 2009; Everaerts, Vriens et al. 2010), which also showed no functional response when exposed to TRPV1 agonists. There are a number of factors that might underlie the inconsistencies concerning the literature on TRPV1 channels in the bladder. Culturing primary cells can have significant effects on gene expression and receptor upregulation. In those studies in which functional response to TRPV1 agonists were absent, the culture time tended to be prolonged relative to those studies which observed TRPV1 mediated responses. Lack of specificity within commercially available TRPV1 antibodies may have also contributed to the controversy (Everaerts, Sepúlveda et al. 2009) and there is also the distinct possibility that rat and mouse urothelial cells express functionally distinct channels.

As described in the previous chapter, receptors for ATP are expressed upon urothelial cells. It has also been shown that interstitial cells express purinergic receptors, and there is significant expression of the homomeric $P2X_3$ receptor on small diameter bladder projecting primary afferents (Chen, Akopian et al. 1995; Dunn, Zhong et al. 2001; O'Reilly, Kosaka et al. 2002), which are thought to co-localise with TRPV1 (Brady, Apostolidis et al. 2004). Thus there are a number of locations within the bladder where receptor co-localisation occurs between TRPV1 and purinergic receptors but the significance of this is yet to be fully explored.

TRPV1 in pathophysiology

The role of the TRPV1 receptor in mediating bladder responses is particularly apparent during inflammation, pain, and bladder disorders where pharmacological blockade or knockout of the TRPV1 receptor is able to mitigate against mechanical hyperactivity.

An essential role for the TRPV1 receptor in mediating sensory afferent discharge was provided following the observation that intravesical capsaicin and resiniferatoxin are effective for treating lower urinary tract symptoms associated with urgency and pain (De Ridder, Chandiramani et al. 1997; Chancellor and de Groat 1999). Patients with idiopathic and neurogenic detrusor overactivity (Silva, Ribeiro et al. 2002; Silva, Silva et al. 2005) with frequency and urge symptoms (Apostolidis, Gonzales et al. 2006; Silva, Silva et al. 2007) show significantly improved sensory urodynamic parameters following intravesical resiniferatoxin and capsaicin treatment as well as a significant reduction in TRPV1 immunoreactivity within the suburothelium

Systemic cyclophosphamide or intravesical acrolein can give rise to bladder mechanical hyperreactivity in WT, but not in $TRPV1^{-/-}$ (KO) mice despite histological evidence of inflammation and cellular damage still being manifest (Wang, Wang et al. 2008). In a lipopolysaccharide model of bladder inflammation, bladder overactivity could be induced in wild-type but not $TRPV1^{-/-}$ (KO) mice (Charrua, Cruz et al. 2007), while inflammatory changes were similar in WT and $TRPV1^{-/-}$ (KO) mice. A distension evoked increase in c-fos in the spinal cord is observed in WT mice but absent in $TRPV1^{-/-}$ (KO) mice, consistent with a role for TRPV1 in noxious sensory signalling and pain related to cystitis. Vizzard et al (2000) showed that cyclophosphamide induced bladder inflammation significantly increased spinal c-fos following distension of the bladder compared to controls and pre-treatment with capsaicin significantly reduced the number of Fos-IR cells induced by bladder distension during cystitis (Vizzard 2000). Bladder cystitis, spinal c-fos, and increased bladder reflexes in rats treated with intraperitoneal cyclophosphamide were significantly attenuated by prior intravesical infusion of

resiniferatoxin (Dinis, Charrua et al. 2004). Indeed, Cefalu et al (2009) showed that bladder overactivity induced by intravesical citric acid was attenuated following TRPV1 blockade and a study using the FIC cat model found functional and morphological changes in DRG neurons sensitive to capsaicin only (Sculptoreanu, de Groat et al. 2005; Sculptoreanu, Artim et al. 2009). These results support the current concept that TRPV1 is important in bladder inflammation.

The role of TRPV1 in inflammation and chemical insult is well documented. The precise mediation of TRPV1 sensitisation in inflammation is not completely understood but depends on protein kinase A and phospholipase C mediated phosphorylation of the receptor (**fig 1.8.1**). This is thought to occur either via inflammatory mediators such as bradykinin, NGF, ATP or PGE_2 (Chuang, Prescott et al. 2001) which activate intracellular messengers, or via the known endogenous activators of TRPV1 such as noxious temperature (>44˚C), acid (<pH 6.0), and anadamide which potentiate each other and open the TRPV1 channel by reducing the heat threshold to normal body temperature levels (Nagy, Sántha et al. 2004; Tominaga and Tominaga 2005). TRPV1 expression on DRG neurons is significantly increased when cultured in the presence of inflammatory mediators (Avelino and Cruz 2006).

These data provide conclusive evidence for the TRPV1 receptor in playing an important role in urinary bladder dysfunction. However, the specific site of action for TRPV1 agonists and antagonists is still unknown. While a direct role on afferent nerve terminals is likely there is also a potential contribution from other urothelial/sub-urothelial cellular targets as well as the detrusor smooth muscle.

The role of TRPV1 within the healthy bladder

TRPV1 is expressed on bladder afferent neurons in rat (Birder, Kanai et al. 2001; Avelino, Cruz et al. 2002), human (Yiangou, Facer et al. 2001; Brady, Apostolidis et al. 2004; Apostolidis, Popat et al. 2005), and mouse (Malykhina, Lei et al. 2012), where a functional response to capsaicin by these afferents has been observed (Daly, Rong et al. 2007). Activation of CSPANS has been shown to induce an ‘efferent’ function associated with the release of neurokinins which are known contractors of detrusor smooth muscle (Maggi, Patacchini et al. 1991) as well as the now known role of capsaicin in directly activating afferent nerve terminals.

The question that still arises, however, is what are the mechanisms for reducing sensory afferent firing following TRPV1 desensitisation? It has been reported that systemic administration of

capsaicin in adult rats results in the degeneration of 90% of axons innervating the ureter (Chung, Schwen et al. 1985), suggesting that the reduction in TRPV1 immunoreactivity and improvement of symptoms could be due to neurotoxicity and loss of mechanosensory afferents and not simply desensitisation of TRPV1. There are a number of unresolved questions. Can TRPV1 act as a mechanosensor to distension and thus denervation of CSPANS leads to direct reduction in mechanosensation? Can TRPV1 desensitisation prevent the 'efferent' release of neurokinins? Can TRPV1 act on non-neuronal targets including the urothelium?

The role of TRPV1 in normal bladder physiology lacks the conclusive evidence that has accumulated for its role in bladder inflammation and pain. However, the widespread distribution of the TRPV1 receptor within the bladder makes it likely that TRPV1 participates in normal bladder function.

Evidence of a TRPV1 role in mediating bladder afferent activity has emerged from a number of sources. $TRPV1^{-/-}$ (KO) mice have a higher frequency of low-amplitude non-voiding contractions during bladder filling and have been reported to have an increase in bladder capacity together with a reduction in spinal cord signalling and reflex voiding (Birder, Nakamura et al. 2002). In another study, spinal c-fos expression in response to bladder distension was similar in $TRPV1^{-/-}$ (KO) and $TRPV1^{+/+}$ (WT) mice (Charrua, Cruz et al. 2007). This lack of effect is consistent with data from Dinnis et al (2004), who observed no effect of capsazepine on bladder reflex activity in normal mice, contrary to the effect seen in inflamed bladders (Dinis, Charrua et al. 2004). Daly et al (2007) recorded multiunit afferent nerve activity from mice and provide solid evidence of a physiological role for TRPV1. Ramp distension of the bladder under physiological conditions causes a graded increase in afferent activity which was significantly attenuated by bath application of capsazepine (Daly, Rong et al. 2007). It was also found that responses were significantly attenuated in $TRPV1^{-/-}$ (KO) mice, thus confirming a role for TRPV1 in afferent signalling.

These results imply that TRPV1 receptors have an essential role in controlling the normal bladder micturition reflex and are not only relevant in times of inflammation and disease. Again however, as is the problem with whole animal systems and knockout mice, it is hard to elucidate the specific actions of TRPV1 within the bladder and to determine if TRPV1 plays a direct role in mechanosensitivity.

In conjunction with the theory of TRPV1 mediated sensory output, there is also a premise that TRPV1 is essential to the initial stages of mechanosensation within the urothelium. TRPV1 activation of cultured urothelial cells has been shown to induce a rise in intracellular calcium and activation of an inward cation current (Charrua, Reguenga et al. 2009; Kullmann, Shah et al. 2009). However, as

mentioned above, this has not been replicated by other investigators using a mouse model (Xu, Gordon et al. 2009; Yamada, Ugawa et al. 2009; Everaerts, Vriens et al. 2010). Importantly in this respect, the presence and activation of TRPV1 has also been shown to alter the release of urothelial mediators. Capsaicin has been shown to release ATP from rat (Sadananda, Shang et al. 2009) and rabbit (Dunning-Davies, Fry et al. 2013) bladder mucosal strips but not porcine bladder (Sadananda, Kao et al. 2012). Exogenous vanilloids evoke nitric oxide release from rat bladder strips, which is reduced by removal of the urothelium or incubation with capsazepine (Birder, Kanai et al. 2001). Also, bladders taken from $TRPV1^{-/-}$ (KO) mice and exposed to TRPV1 agonist failed to show an increase in intracellular calcium or nitric oxide release (Birder, Nakamura et al. 2002). Furthermore, urothelial ATP release from both whole mouse bladder and cultured urothelial cells was blunted in the $TRPV1^{-/-}$ (KO) mice (Birder, Nakamura et al. 2002).

The role of TRPV1 as a direct mechanosensor to bladder distension has been proposed but has proven hard to determine conclusively due to the multiple levels of interaction within the bladder wall eg, urothelial release Vs muscle compliance Vs direct afferent discharge and this will be a major focus of this study.

Is there a link between TRPV1 and P2X?

There is evidence that activation of $P2X_3$ sensitive afferents is essential to normal voiding behaviour and that there is interaction between purinergic and TRPV1 receptors within the bladder. There is also evidence that the effects of $TRPV1^{-/-}$ (KO) can be mediated through interactions with the purinergic system, either at the level of the urothelium, or the sensory afferents.

Many reports suggest that endogenous ATP is released from the urothelium during bladder distension (Ferguson, Kennedy et al. 1997; Collins, Daly et al. 2013) and acts on sub-urothelial afferent nerves to trigger afferent activity. There is significant purinergic receptor expression on bladder afferents (Vlaskovska, Kasakov et al. 2001) and this expression is principally P2X on small diameter sensory nerves (Chen, Akopian et al. 1995; Bradbury, Burnstock et al. 1998; Burnstock 2000), with $P2X_3$ receptor immunoreactivity predominantly seen within bladder projecting afferents (Cockayne, Hamilton et al. 2000). There is growing evidence of an essential role for the $P2X_3$ receptor in normal and pathological bladder function. ATP is significantly involved in the activation of pelvic nerve afferents arising from the rat urinary bladder (Namasivayam, Eardley et al. 1999) and has been shown, at high concentrations, to initiate bladder overactivity (Pandita and Andersson 2002). The

purinergic agonist αβMe-ATP increases afferent discharge in both high-threshold nociceptive and low-threshold physiological mechanosensory bladder afferents (Rong, Spyer et al. 2002). $P2X_3$-null mice exhibit a marked urinary bladder hyporeflexia, characterized by decreased voiding frequency and increased bladder capacity (Cockayne, Hamilton et al. 2000) whilst $P2X_3^{-/-}$ and $P2X_{2/3}^{-/-}$ (KO) mice show reductions in bladder afferent firing (Vlaskovska, Kasakov et al. 2001; Cockayne, Dunn et al. 2005).

Both $P2X_3$ and TRPV1 immunoreactive fibers have been identified on bladder afferents in the sub-urothelium (Cockayne, Hamilton et al. 2000; Lee, Bardini et al. 2000; Yiangou, Facer et al. 2001) and colocalisation of TRPV1 with P2X receptors has been confirmed within a large proportion of dorsal root ganglion neurons (Guo, Vulchanova et al. 1999). There is also considerable functional evidence that a large proportion of capsaicin sensitive DRG neurons also respond to ATP and αβMe-ATP (Piper and Docherty 2000; Dang, Bielefeldt et al. 2005) and co-immunoprecipitation studies demonstrate the physical association of TRPV1 and $P2X_3$ receptors on DRG neurons (Stanchev, Blosa et al. 2009).

$P2X_3$ immunoreactivity on bladder projecting neurons is significantly increased in patients with neurogenic detrusor overactivity (Brady, Apostolidis et al. 2004) and is significantly reduced in those patients who responded positively to intravesical resiniferatoxin suggesting there is functional colocalisation between the TRPV1 and $P2X_3$ receptors on bladder afferent nerve terminals (Brady, Apostolidis et al. 2004). Botulinum toxin injected into the bladder wall also reduces both TRPV1 and $P2X_3$ immunoreactivity (Apostolidis, Popat et al. 2005), and there is a significant correlation between the reduction in TRPV1 and $P2X_3$ immunoreactivity in nerve fibers with the frequency of urgency episodes of patients, confirming a sensory role for both receptors.

Along with a proposed role for TRPV1 and P2X coordination on afferent nerves, there is evidence that the TRPV1 receptor is involved in urothelial release of ATP in responses to distension, with $TRPV1^{-/-}$ (KO) mice showing a marked reduction in ATP release as described in more detail above. As such, it is probable that $P2X_3$ receptors localised on nerves in the suburothelium perform a mechanosensory role and we propose that the TRPV1 receptor could play an important role in their modulation.

So far there have been a number of studies investigating the importance of TRPV1 and P2X in peripheral control of the micturition reflex. Co-localisation of these receptors within bladder tissues raises the possibility that they play an important role in the response to mechanical stimulation and the micturition reflex in models of inflammation. As yet there have been no studies investigating a sensory transduction role based on a functional interaction between TRPV1 and P2X receptors under

non-pathological conditions. This study will investigate the afferent response to P2X stimulation in a pelvic afferent nerve model using pharmacological blockade and genetic deletion of the TRPV1 receptor. This study will go further into examining the role of TRPV1 at both the whole organ level, as well as in isolated urothelial cells and DRG neurons to elucidate the interactions alluded to in previous studies about the origins and importance of TRPV1 and P2X receptors on bladder function.

4.2 EXPERIMENTAL PROTOCOLS

The main methodology is detailed in chapter 2. Here the emphasis is on the specific protocols that were employed to address the role of TRPV1 in P2X mediated bladder afferent signalling.

Intravesical pressure and afferent nerve recording.

Bladder contraction

In order to measure contractile responses of detrusor smooth muscle and corresponding afferent nerve activity, bladders were perfused intravesically with 0.9% saline to a maximum pressure of 12mmHg following control ramp distensions. The syringe pump was stopped but the outflow tap remained closed, keeping a set volume within a slightly distended bladder and the bladder muscle was given time to accommodate this volume and become stable (**fig 2.4.1**). Responses to αβMe-ATP (300nM, 1μM, 3μM, 10μM, 30μM, 100μM) were obtained. For contraction responses to compare $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice αβMe-ATP (30μM) and 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP) (100μM) were diluted from stock solutions dissolved in dH_20, diluted into krebs solution and applied via bolus dose of 900μl to the experimental bath of volume 30ml. In experiments in which multiple applications of αβMe-ATP were applied in the same experiment, a washout period of 45minutes between each dose ensured receptors were not desensitised.

The responsiveness of bladder afferents to αβMeATP (30μM) was also examined in calcium free / high magnesium medium to examine the direct response to afferent nerve stimulation independent of bladder contraction. As previously discussed, experiments were set up in a normal calcium containing Krebs and were left to equilibrate for 30 minutes. The perfusate was then switched to the Ca^{2+} free medium and preparations were left for a further 30 minutes (NaCl 118mM, KCL 4.75mM, NaH_2PO_4 1.0mM, $NaHCO_3$ 25mM, $MgSO_4$ 1.2mM, glucose 11mM, EDTA 1mM, Mg^{2+} 6mM as used previously by Zagorodnyuk et al*.,* (2006).

Capsazepine (CPZ) (Stock @1mg/ml DMSO) 10μM was diluted in krebs solution and applied via the perfusion pump (5ml/min) to the experimental bath for 30min prior to agonist application.

Ramp distensions

All ramp distensions in this study were performed at a rate of 100µl/min to a maximal pressure of 40mmHg based on pilot experiments and previously published literature (Rong, Spyer et al. 2002; Daly, Rong et al. 2007). Repeat ramp distensions at 100µl/min elicit reproducible afferent nerve and pressure responses (**fig 4.2.1**).

Intravesical drug administration

αβMe-ATP (30µM) or saline control (0.9% saline + vehicle) was infused into the bladder lumen via the syringe pump. The syringe pump provided constant infusion of drug or saline at 30µl/min with the outflow tap open to prevent bladder distension and in order to ensure that the drug concentration was stable during the subsequent ramp distension. Preliminary experiments were conducted at higher flow rates but the bladder could not empty at an equivalent rate and thus volume, and pressure inside the bladder increased with infusion rates above 30µl/min. Drug infusion immediately following the prior control distension for a minimum of 7 minutes before distension was repeated with the drug present.

Periods of control or agonist infusion without bladder distension were analysed for spontaneous baseline afferent discharge having calculated that the dead space between the syringe pump and the bladder was 120µl and a latency of response would be 4 minutes.

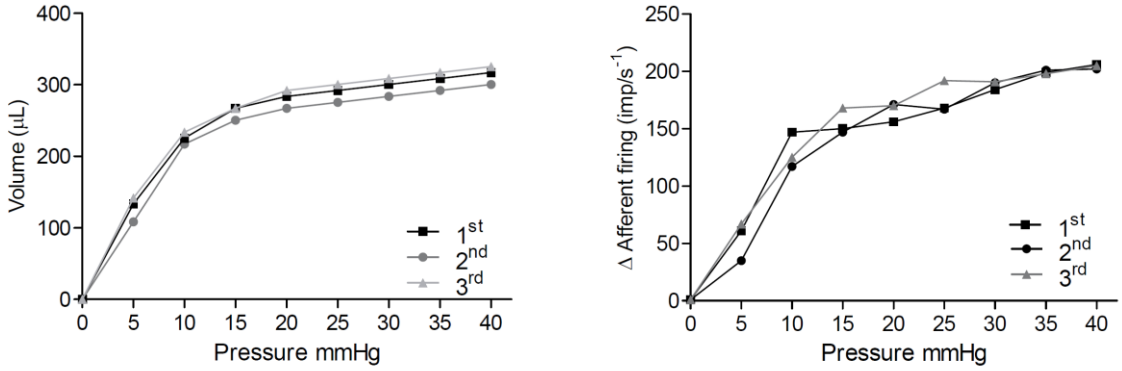

Fig 4.2.1. *Pressure/volume and pressure/nerve responses to three repeat distensions at 100µl/min prior to the start of an experiment. The compliance and afferent response to distension are reproducible.*

qPCR

qPCR experiments were performed as described in the major methods section. DRG neurons and PMUC's were isolated from mice and cultured for 24hrs at 37°C. mRNA was harvested, converted to cDNA and RT-qPCR was performed using SYBR-Green fluorescence with the TRPV1 primer at a Tm of 59°C.

Gene		Sequence '5-3'	Product	Position	Tm	Chromosome
TRPV1	Forward	CAAGGCTCTATGATCGCAGG	198bp	575-772	58.2	11
	Reverse	GAGCAATGGTGTCGTTCTGC			59.8	

Table 4.2.1 *Primer sequence, product size, gene loci, and Tm for purinergic receptors investigated in this study.*

Mediator release

Mediator release experiments were performed with infusion set at 100µl/min and to a maximal pressure of 40mmHg to replicate the conditions in which the afferent nerve recordings were performed.

Basal samples of intraluminal contents were taken during a 7 minute infusion of vehicle/agonist @30µl/min with the outflow tap open to prevent a build up of volume and bladder pressure, the outflow tube was placed into a sterile eppendorf tube placed on dry-ice to prevent post-hoc degradation of substrates. Immediately following ramp distensions, the syringe pump was stopped and the outflow catheter was placed into a sterile eppendorf tube placed in dry-ice to collect the intravesical contents whilst the bladder was left to empty passively.

Intravesical contents from basal and distension (stretch) in control and drug treated preparations were assayed for ATP (Molecular Probes® A22066) and ACh (Molecular Probes® A12217) using commercially available kits. Unknowns were calculated from a standard curve of known concentrations with blank values subtracted.

Calcium imaging

The calcium imaging protocol is described in more detail in the major methods chapter.

Calcium imaging was performed using an excitation wavelength of 510nm light and ratios were calculated from emissions at 350/380nm or 340/380nm with background fluorescence deducted. ATP concentrations used in urothelial concentration-response curves were 1mM, 100µM, 10µM, 1µM, 100nM 10nM using serial dilutions. αβMe-ATP (30µM) and Ionomycin (5µM) were diluted from stock into HEPES solution and applied using a gravity perfusion system at a rate of 1.5ml/min for 60s and 5 minutes respectively. Data are presented as a ratio of fluorescence Rf (350/380), or Rf (340/380), N= number of experiments, n= number of cells.

4.3 THE CONTRIBUTION OF TRPV1 TO BLADDER AFFERENT NERVE ACTIVITY

As the bladder filled with saline there was a graded increase in intraluminal pressure and a concurrent increase in the afferent nerve firing. The results in **fig 4.3.1** show that the afferent nerve response to distension in TRPV1$^{-/-}$ (KO) mice was significantly reduced compared to age matched WT control mice ($p \leq 0.01$, n=9, Two-way ANOVA, Bonferroni multiple comparisons post-hoc test). This difference in afferent nerve firing becomes more apparent as the pressure is increased above 10mmHg and suggests a mechanism whereby the TRPV1 receptor is involved in mediating a small component of the afferent nerve responses to distension. The compliance of the detrusor smooth muscle to an increase in volume was unaffected by TRPV1 knockout (**fig 4.3.2**). Peak afferent discharge was found to occur at the highest distension pressure for all preparations.

There is a variable degree of spontaneous afferent nerve firing when the bladder was empty with occasional bursts of firing. In experiments in which such spontaneous activity was present, there was a trend for reduced levels of firing in the TRPV1$^{-/-}$ (KO) mice but this failed to reach statistical significance (**fig 4.3.3, fig 4.3.4**).

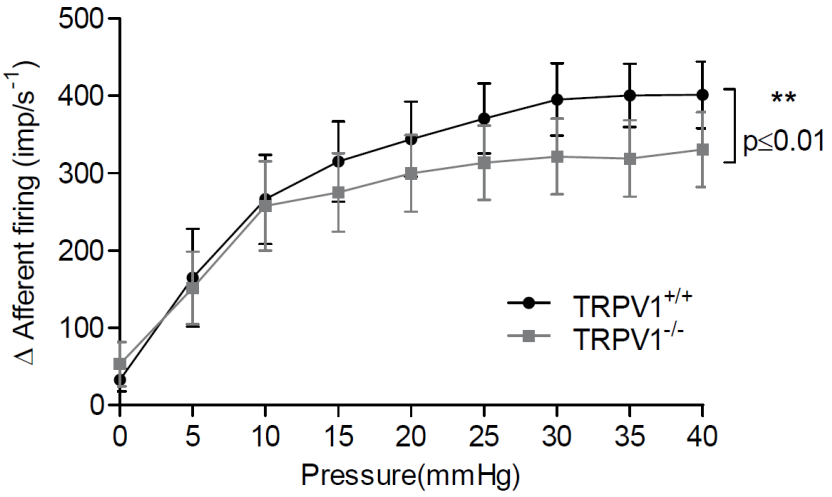

Fig 4.3.1 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with saline at a rate of 100µl/min in TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice (**p≤0.01, n=9).*

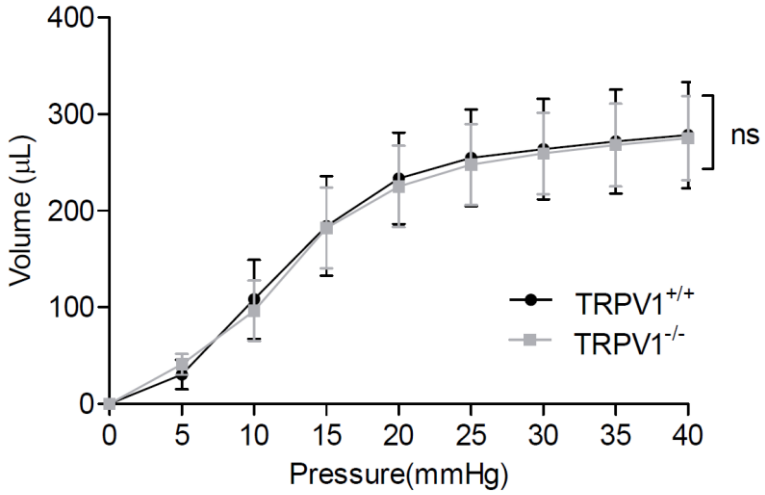

Fig 4.3.2 *Mean±(SEM) pressure/volume relationship within the bladder when distended with saline at a rate of 100µl/min in TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice (n=9).*

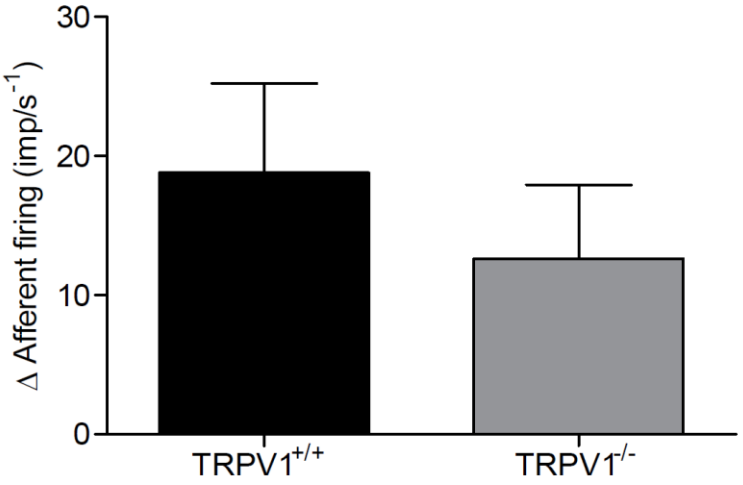

Fig 4.3.3 *Mean±(SEM) peak baseline spontaneous afferent activity recorded over 100s whilst the bladder was empty in TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice (n=7).*

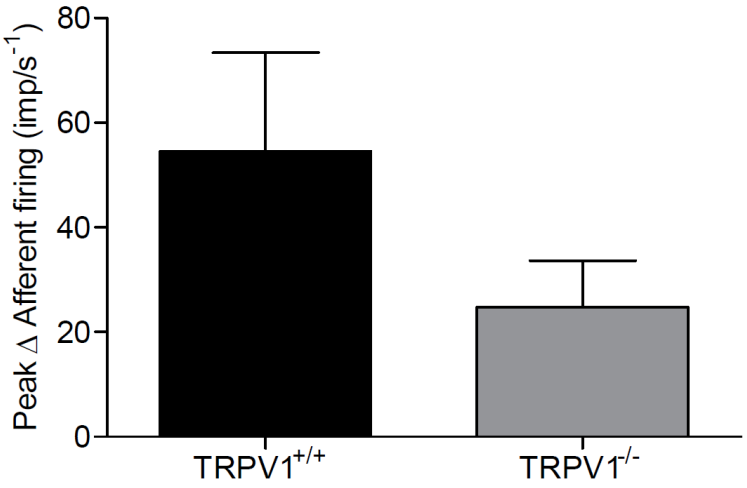

Fig 4.3.4 *Mean±(SEM) peak baseline spontaneous afferent activity recorded during 100s whilst the bladder was empty in TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice (n=7).*

4.4 THE ROLE OF TRPV1 IN P2X MEDIATED AFFERENT SIGNALLING

Bladder afferent nerves respond to the purinergic agonist αβMe-ATP with an initial sharp increase in afferent nerve discharge (**fig 4.4.1**) which immediately begins to decrease in amplitude even in the continued presence of the agonist. Afferent nerve responses were concentration-dependent with a pEC50 of 7.57±0.5μM (n=6). An experimental trace from a typical experiment in which increasing concentrations of αβMe-ATP were applied to the bladder can be seen in **fig 4.4.2**. Upon application of αβMe-ATP there was also a concurrent contraction of the detrusor smooth muscle. This data confirms that the smooth muscle and afferent nerves serving the bladder are sensitive to activation by purinergic agonists.

Application of a single dose of bath applied αβMe-ATP (30μM) initiates a significant increase in afferent nerve activity (**fig 4.4.3**). The afferent response to αβMe-ATP is characterised by an initial rapid component followed by a period of more sustained firing before returning to baseline over 2-3 minutes which is accompanied by a corresponding detrusor smooth muscle contraction as indicated by an increase in intravesical pressure (**fig 4.4.4**). The responses to αβMe-ATP (30μM) were reproducible with sufficient time allowed between agonist administrations to allow receptor resensitisation (**Fig 4.4.4**). Maximum responses (**fig 4.4.5**), and time course profiles (**fig 4.4.3**) to αβMe-ATP (30μM) were not significantly different for the two additions of agonist ($p \geq 0.05$, n=9, two way ANOVA, Bonferroni multiple comparisons post-hoc test).

Afferent nerve firing during the αβMe-ATP (30μM) response time course was significantly attenuated in TRPV1$^{-/-}$ (KO) mice compared to WT littermates ($p \leq 0.0001$, n=9: Two-way ANOVA, Bonferroni multiple comparisons post-hoc test) (**fig 4.4.6**). These results suggest that TRPV1$^{-/-}$ (KO) mice have reduced afferent nerve sensitivity to purinergic stimulation. In order to examine this interaction further, the time course responses to purinergic agonists in WT mice were also examined in the absence and presence of the TRPV1 receptor antagonist capsazepine (10μM) (**fig 4.4.7**). Capsazepine (10μM) significantly reduced the duration of the responses to αβMe-ATP (30μM) in a similar manner to that seen in the knockout ($p \leq 0.0001$ n=9: Two-way ANOVA, Bonferroni multiple comparisons post-hoc test). An experimental trace showing αβMe-ATP induced afferent nerve firing and detrusor contraction before and following capsazepine (10μM) in WT mice can be seen in **fig 4.4.8**.

Peak responses to αβMe-ATP in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice with capsazepine are summarised in **fig 4.4.9**. Bath applied αβMe-ATP (30μM) caused a significant rise in afferent nerve activity reaching a peak of 136.8±30.8imp s^{-1} (n=9) in control (WT) mice. The response to αβMe-ATP (30μM) was significantly reduced in TRPV1$^{-/-}$ (KO) mice (47.4±16.6imp s^{-1} p≤0.05, n=9) similar to that seen with prior capsazepine (10μM) incubation (63.16±27.8imp s^{-1} p≤0.05, n=9, one-way ANOVA Bonferroni multiple comparisons post-hoc test)

These results indicate that there is a significant decrease in purinergic evoked afferent nerve discharge, both in terms of magnitude and duration of the responses, with either pharmacological blockade or genetic knock-out of the TRPV1 receptor.

Further analysis of the data for αβMe-ATP responses indicated that there is also a change in the kinetics of the afferent nerve discharge (**fig 4.4.10**). Bath applied αβMe-ATP (30μM) induces peak afferent nerve firing in WT mice after 18±3.2s. TRPV1$^{-/-}$ (KO) mice (64.4±10.4s p≤0.001, n=9) and WT mice with capsazepine (10μM) (51.1±11.6s p≤0.05, n=9, one-way ANOVA, Bonferroni multiple comparisons post-hoc test) showed a significant increase in the time taken to reach peak afferent activity.

These results indicate there is a functional link between the TRPV1 receptor and purinergic evoked afferent nerve responses in the mouse bladder. In order to examine this interaction further it was first essential to investigate if this was due to a general change in neuronal excitability, or if it is a specific interaction between the TRPV1 and P2X receptors.

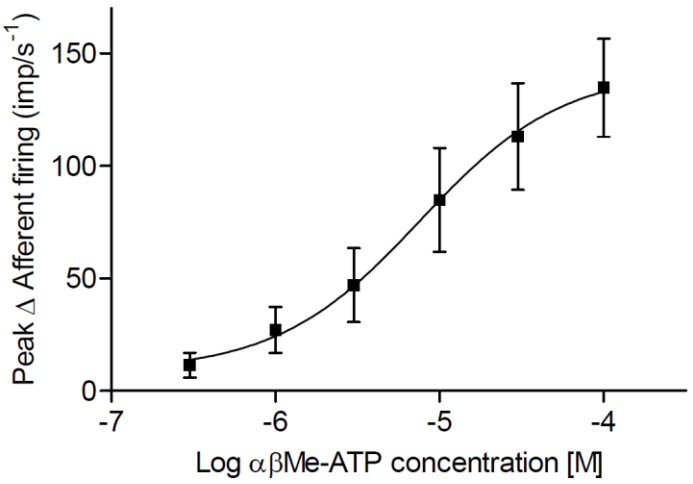

Fig 4.4.1 *Mean±(SEM) concentration-response curve of afferent nerve activity to αβMe-ATP (300nM-100µM, n=6).*

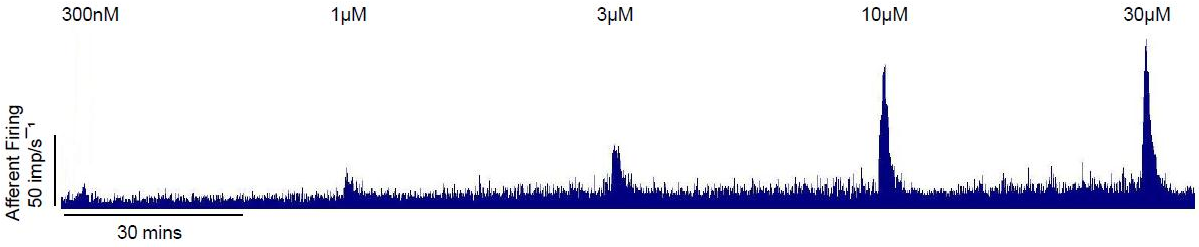

Fig 4.4.2 *Experimental trace showing afferent nerve activity in response to increasing concentrations of αβMe-ATP. A 45 minute washout was allowed between each successive dose to prevent receptor desensitisation.*

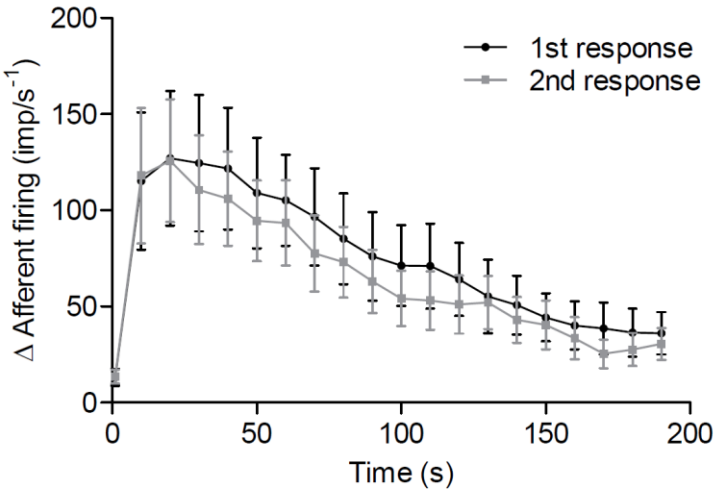

Fig 4.4.3 *Mean±(SEM) afferent nerve activity over the duration of the response to repeated applications of αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) mice from the same experiment (n=9).*

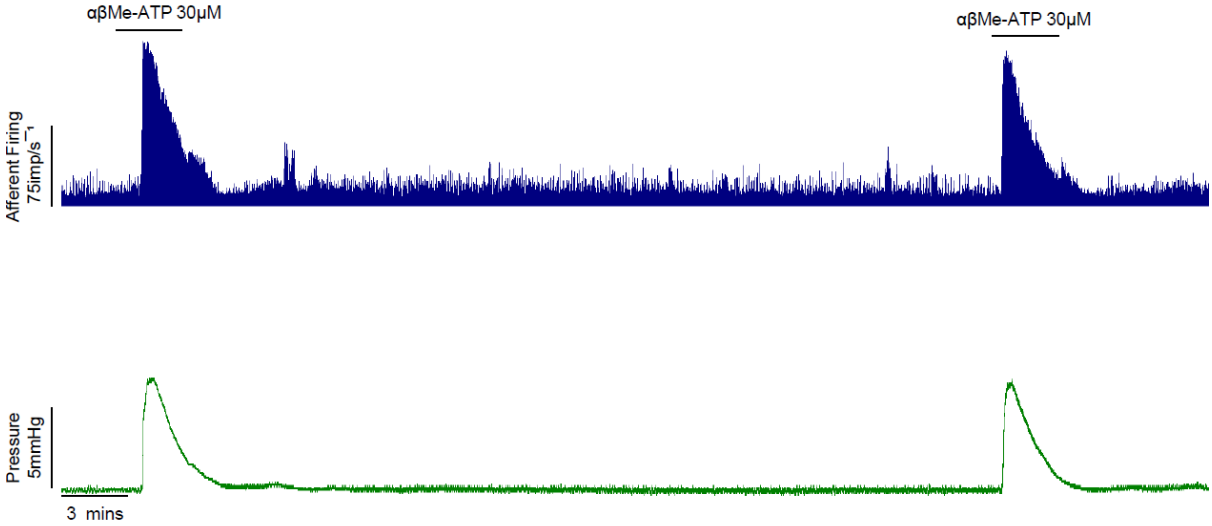

Fig 4.4.4 *Experimental trace showing the reproducibility of afferent nerve and intravesical pressure responses to repeat application of αβMe-ATP (30μM) in control mice. These experiments were performed in a bladder predistended to 12mmHg in order to accurately measure intravesical pressure responses.*

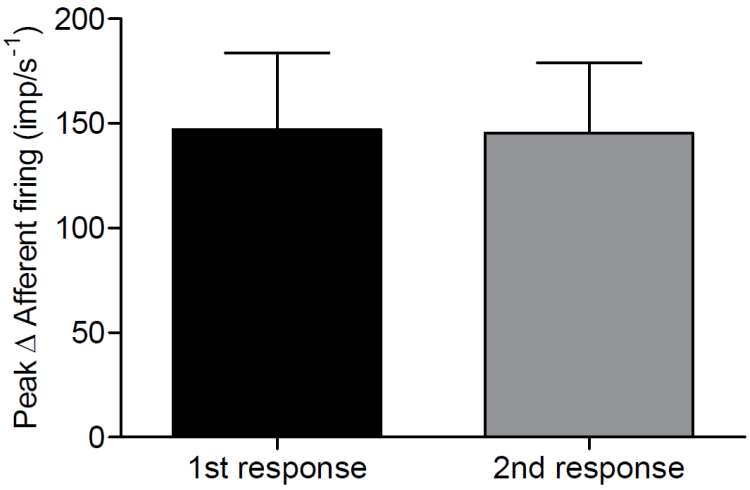

Fig 4.4.5 *Mean±(SEM) peak afferent firing in response to repeat application of αβMe-ATP (30μM) in control mice (n=9).*

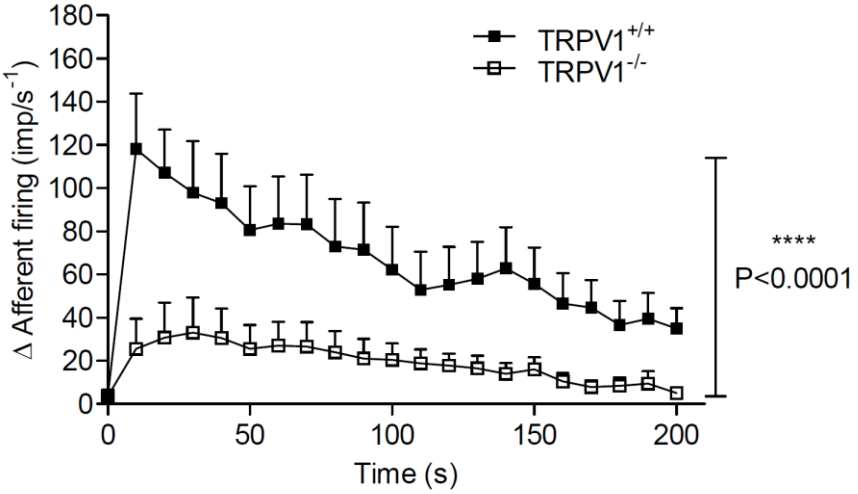

Fig 4.4.6 *Mean±(SEM) afferent nerve activity over the duration of the response to applications of αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (****p≥0.0001, n=9).*

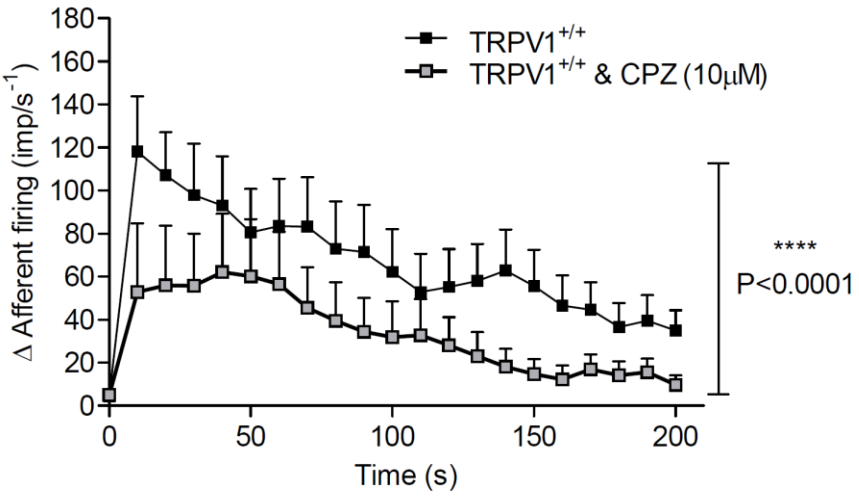

Fig 4.4.7 *Mean±(SEM) afferent nerve activity over the duration of the response to αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) mice in the presence and absence of capsazepine (10μM) (****p≥0.0001, n=9).*

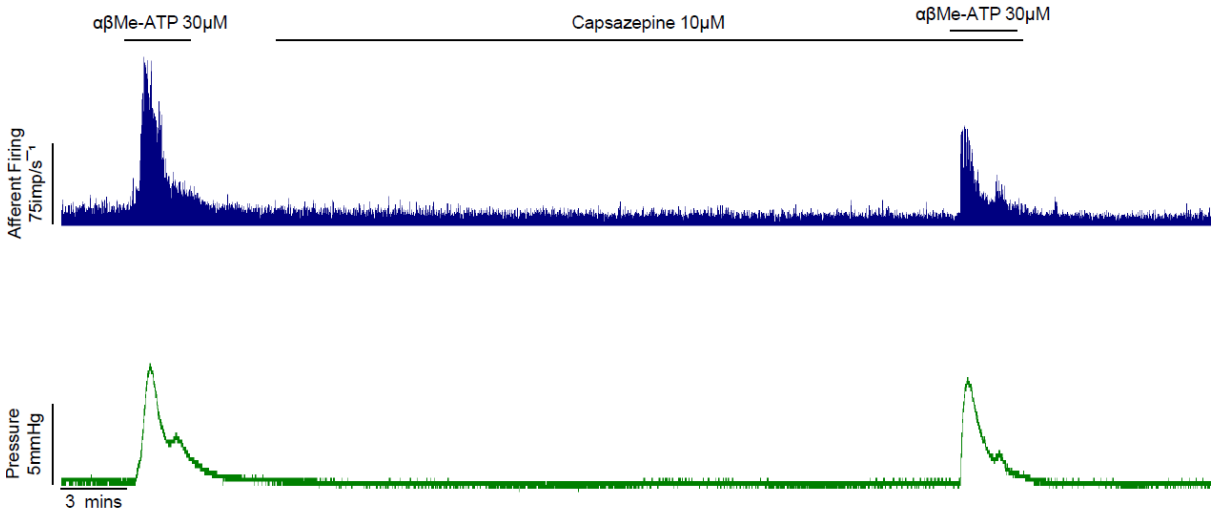

Fig 4.4.8 *Experimental trace showing afferent nerve and intravesical pressure responses to repeat application of αβMe-ATP (30μM) in control mice prior to and following incubation with the TRPV1 antagonist capsazepine (10μM).These experiments were performed in a bladder predistended to 12mmHg in order to accurately measure intravesical pressure responses. Note that while afferent response is attenuated, the pressure profile is similar after capsazepine.*

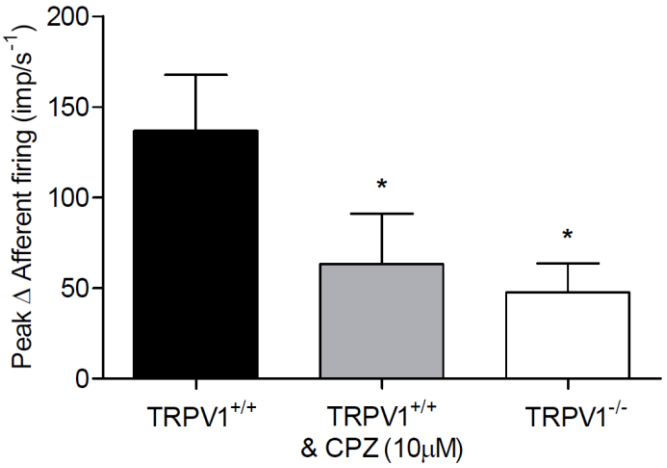

Fig 4.4.9 *Mean±(SEM) peak afferent nerve responses to αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) in the presence and absence of capsazepine (10μM), and TRPV1$^{-/-}$ (KO) mice. (*p≤0.05 compared to TRPV1$^{+/+}$, n=9).*

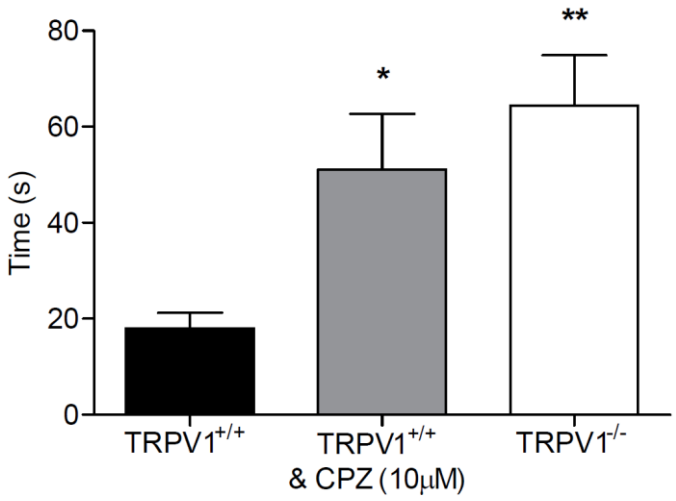

Fig 4.4.10 *Mean±(SEM) time taken to reach maximum afferent nerve impulse frequency in response to αβMe-ATP (30μM). (**p≤0.01, *p≤0.05 compared to TRPV1$^{+/+}$, n=9).*

4.5 IS THE EFFECT OF TRPV1 COUPLED TO P2X MEDIATED AFFERENT FIRING?

To determine if the decreased afferent responses to αβMe-ATP were due to a general change in neuronal excitability, peak afferent nerve response to the nicotinic agonist DMPP (100μM) in $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice were examined (**fig 4.5.1**). DMPP (100μM) applied to bladders from WT mice caused a significant increase in peak afferent firing 139.1 ± 46.1imp s^{-1} (n=12). Knockout of the TRPV1 receptor had no significant effect on the peak afferent response to DMPP 157.2±52.3imp s^{-1}, ($p \geq 0.05$ n=7, unpaired t-test). The maintenance of nicotinic evoked afferent responses in both $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice suggest that changes seen in purinergic activation of bladder afferent nerves by removal or blockade of the TRPV1 receptor are not due to a general decrease in afferent nerve excitability.

As shown above, increases in afferent nerve firing following αβMe-ATP (30μM) occur with a concurrent contraction of the detrusor smooth muscle which is known to influence afferent activity. In order to determine if the changes in afferent nerve firing due to pharmacological blockade or knock-out of the TRPV1 receptor are a result of altered detrusor muscle activity, the detrusor contraction responses were also examined. There was no significant difference in the contraction responses of $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mouse bladders (**fig 4.5.2**), as measured by a change in intravesical pressure (12.4±1.6 Vs 12.06±1.3mmHg $p \geq 0.05$, n=9). Similarly, capsazepine (10μM) also failed to alter contraction responses to αβMe-ATP (30μM) in $TRPV1^{-/-}$ (KO) mice 12.06±1.3 Vs 12.5±3mmHg ($p \geq .005$, n=4, One-way ANOVA, Bonferroni multiple comparisons post-hoc test). The results from **fig 4.3.2** also show that there is no change in the compliance of the detrusor smooth muscle in $TRPV1^{-/-}$ (KO) mice.

These results suggests that any changes in afferent nerve discharge are not as a result of a change in detrusor muscle activity and can be attributed to changes associated with afferent nerve firing.

A further test of this hypothesis was to remove the smooth muscle contraction component of the αβMe-ATP response. This was achieved by repeating experiments in a calcium free krebs solution with or without capsazepine (10μM) (**fig 4.5.3**). A significant reduction in the peak afferent nerve response to αβMe-ATP (30μM) was still observed following capsazepine (10μM) in calcium-free buffer (86.6±16.3imp^{-s} Vs 53.6±10.8imp^{-s}, $p \leq 0.05$, n=7, unpaired t-test).

The results suggest that there is a significant interaction between a functional TRPV1 receptor and the purinergic component of afferent nerve activity. There is no difference in the contraction responses of the detrusor smooth muscle and thus the interaction is likely to be at the level of the afferent nerves. These experiments have concentrated on the application of bath applied purinergic agonists, however, with relevance to a more physiological difference, the alterations in afferent nerve responses to distension in TRPV1 KO mice seen in **fig 4.3.1** could theoretically occur from a number of sources in any part of the bladder wall.

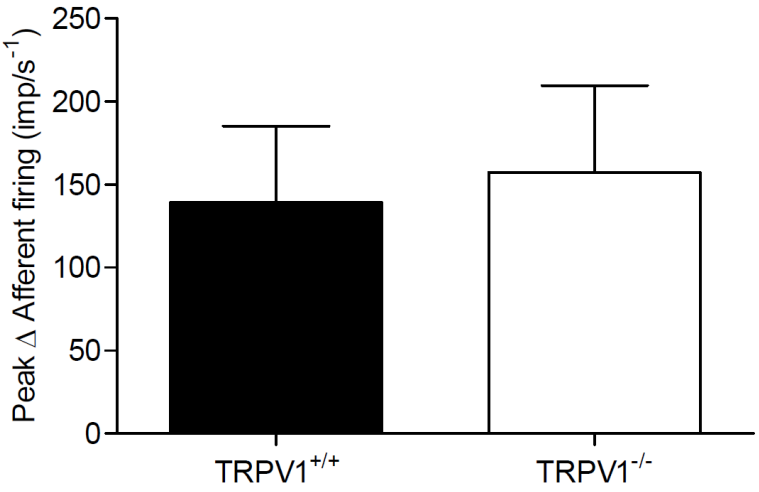

Fig 4.5.1 *Mean±(SEM) Peak afferent nerve response to bath applied nicotinic agonist DMPP (100µM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice* (n=7).

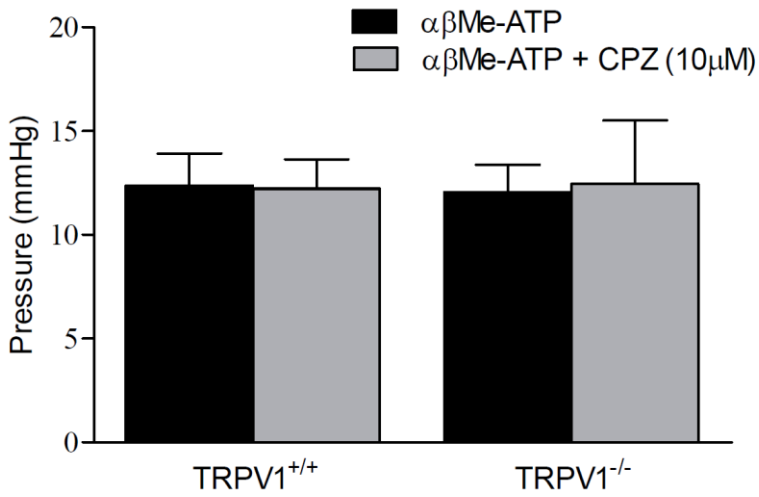

Fig 4.5.2. *Mean±(SEM) Peak intravesical pressure responses to αβMe-ATP (30µM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice with or without capsazepine (10µM). (n=9, 9, 9, 4)*

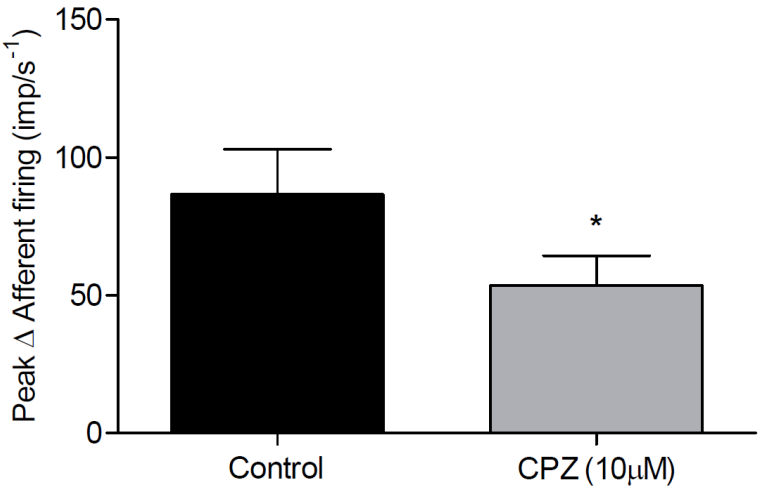

Fig 4.5.3 *Mean±(SEM) peak afferent nerve responses to bath applied αβMe-ATP (30μM) in a calcium free modified krebs solution with or without capsazepine (10μM). (*p≤0.05, n=7).*

4.6 THE EFFECT OF INTRAVESICAL αβMe-ATP ON AFFERENT NERVE ACTIVITY

The following experiment observed the effects of intravesical perfusion of αβMe-ATP (30μM) on spontaneous afferent activity. Intravesical perfusion of αβMe-ATP (30μM) significantly increased spontaneous afferent firing ($p \leq 0.05$, n=6, paired t-test, **fig 4.6.2**). The experimental trace in **fig 4.6.1** shows an example of the relationship between the raw nerve activity and the rate histogram of afferent nerve activity imp/s^{-1}. There is an increase in afferent nerve activity with no change in intravesical pressure. The afferent nerve response profile is different from that with bath application of αβMe-ATP (30μM) and could represent diffusion across the urothelial barrier, or secondary actions following receptor binding on urothelial cells.

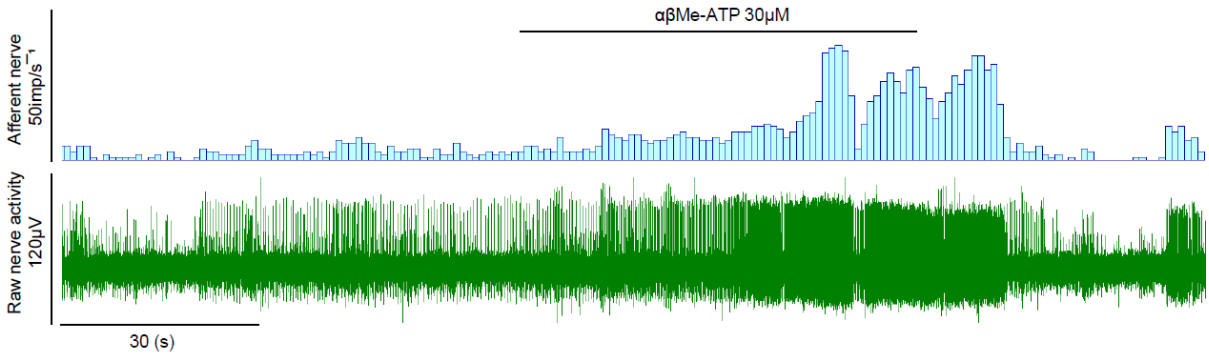

Fig 4.6.1 *Experimental trace representing the effects of intravesical instillation of αβMe-ATP (30μM) on baseline spontaneous afferent activity. The upper trace is a rate histogram of nerve activity (imp/s^{-1}), the middle trace is the raw nerve activity, and the bottom trace shows intravesical pressure.*

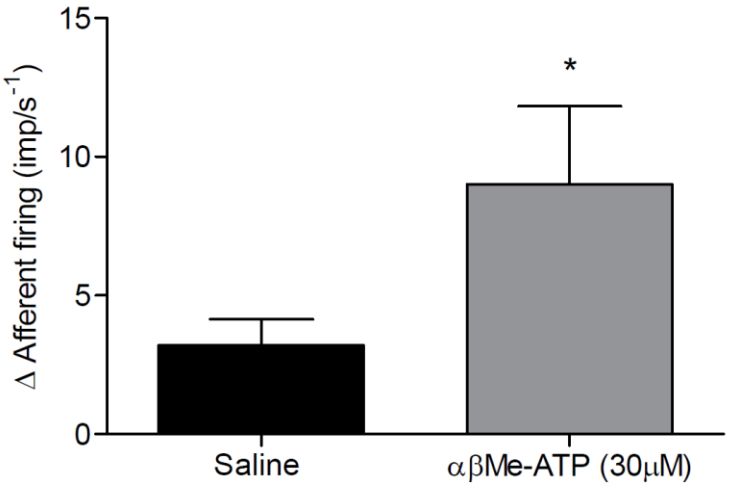

Fig 4.6.2 *Mean±(SEM) average baseline spontaneous afferent activity recorded over 100s prior to, and following intravesical instillation of* αβMe-ATP (30μM) (*p≤0.05, n=6).

4.7 UROTHELIAL AND DRG RESPONSES IN TRPV1$^{+/+}$ (WT) AND TRPV1$^{-/-}$ (KO) MICE

The following experiments have explored further the idea that a variety of cell types could be contributing to the interaction between TRPV1 and mechanosensitivity seen in fig **4.3.1.** Specifically, these experiments were conducted to explore on the one hand the possibility of a functional contribution of TRPV1 in urothelial cells and on the other hand to examine the sensitivity of DRG neurons from TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice in isolation from the urothelium.

Relative mRNA expression of the TRPV1 receptor in isolated PMUCs and DRG neurons is shown (**fig 4.7.1**). mRNA for the TRPV1 receptor is expressed in both PMUCs and DRGs. However, the levels of expression were significantly greater in DRG neurons than for PMUCs ($p \leq 0.01$, n=3, unpaired t-test).

In order to investigate the role of the TRPV1 receptor in responses to the purinergic agonist ATP in urothelial cells, a concentration response curve of primary mouse urothelial cells to ATP (10nM-1mM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice was constructed (**fig 4.7.2**). Data normalised to percentage of maximal response show PMUCs respond in a concentration-dependent manner to the purinergic agonist ATP with an pEC50 values of 3.49±0.77μM and 4.2±0.74μM in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) respectively (N=6, n=360, 470). There was no significant difference between pEC50 responses to ATP of urothelial cells from TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice and no change in the concentration of ATP required to elicit a maximal intracellular calcium response (100μM). While the sensitivity to ATP was similar between TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice, the magnitude of the calcium-signal was different. Calcium fluorescence data is presented (**fig 4.7.3**) showing that PMUC's from TRPV1$^{-/-}$ (KO) mice have a significantly potentiated response to ATP ($p \leq 0.001$, N=6, n=360, 470, two-way ANOVA, Bonferroni multiple comparisons post-hoc test) indicating that the TRPV1 receptor, or lack thereof, is able to influence purinergic signalling in PMUCs. Paradoxically, the calcium-response is greater in the absence of TRPV1.

The presence of a functional TRPV1 receptor within PMUCs was further tested by measuring intracellular calcium responses to the application of the TRPV1 agonist capsaicin (1μM) (**fig 4.7.4**). PMUCs did not show a rise in intracellular calcium in response to capsaicin (1μM) despite responses to application of ATP being observed (N=3, n=57).

Further investigation into the role of the TRPV1 receptor in mediating bladder distension responses was achieved through analysis of the luminal contents for ATP and acetylcholine prior to, and following bladder distension.

The effect of bladder distension on the release of acetylcholine (**Fig 4.7.5**) shows a consistent release of acetylcholine from the bladder at rest (basal) and a non-significant increase in release during bladder distension (0.35±0.13µM, basal Vs 0.50±18µM stretch). Knock-out of the TRPV1 receptor had no effect on the release of acetylcholine at rest or during bladder distension. Basal: 0.36±0.11 (KO) Vs 0.35±0.13 (WT), Stretch: 0.57±.11 (KO) Vs 0.50±18µM (WT) ($p \geq 0.05$ compared to WT basal and stretch, n=6, two-way ANOVA, Bonferroni multiple comparisons post-hoc test).

The effect of bladder distension on the release of ATP (**fig 4.7.6**) shows ATP is released into the bladder lumen at low levels during rest, and at significantly higher levels during distension (2.5±0.3 Vs 14.4±3.4nM $p \leq 0.001$ n=6) in TRPV1$^{+/+}$ (WT) mice. In TRPV1$^{-/-}$ (KO) mice, there was no difference in basal release of ATP (2.5±0.3 Vs 3.1±0.8nM) but a significant decrease in stretch evoked ATP release from the bladder into the lumen when compared to WT mice (14.4±3 Vs 4.1±0.9nM $p \leq 0.01$ n=6, one-way ANOVA, Bonferroni multiple comparisons post-hoc test). The results show that knock-out of the TRPV1 receptor was able to significantly inhibit distension evoked ATP release.

Pelvic DRG neurons were able to respond to αβMe-ATP (30µM) with a variable increases in intracellular calcium (**fig 4.7.8**) which rose rapidly to a peak before a swift reduction in response towards baseline. The individual cell responses and variability can be seen in **fig 4.7.7**. Application of αβMe-ATP (30µM) to DRGs caused desensitisation of receptors on DRG neurons as shown by the significant reduction following repeat application of αβMe-ATP following a 5 minute washout in HEPES. The second response to αβMe-ATP shows a 69.5±5% reduction in maximal response ($p \leq 0.0001$, N=4, n=32, Wilcoxon matched-pairs signed rank test, **fig 4.7.9**).

The measurement of intracellular calcium levels within DRG neurons (**fig 4.7.10**) showed that DRG neurons from WT mice respond to the TRPV1 agonist capsaicin (300nM) with an increase in intracellular calcium which varied considerably between individual cells. DRG neurons from TRPV1$^{-/-}$ (KO) mice failed to respond to capsaicin (300nM). DRG neurons from both TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) responded to ATP (100µM) with an increase in intracellular calcium. These results confirm the knockout of the TRPV1 receptor by removing the response to extracellular capsaicin (300nM) and that a proportion of pelvic DRG neurons are sensitive to both TRPV1 and purinergic agonists.

In order to investigate the hypothesis that there is a functional link between TRPV1 and P2X receptors on the afferent neurons innervating the bladder, and that this link is responsible for

reduced afferent discharge in distension responses as well as responses to αβMe-ATP (30μM) in the in-vitro afferent model, isolated DRG neurons from TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice were exposed to αβMe-ATP (30μM) and the peak intracellular calcium response was measured (**Fig 4.7.12**). Data was normalised to percentage of maximum ionomycin induced calcium influx to overcome intra-experimental variability. There was no significant difference in maximal αβMe-ATP (30μM) induced calcium influx in DRG neurons 24.4±2.4% Vs 28.1±3.2% in WT and KO mice respectively ($p \geq 0.05$, N=12, n=80, unpaired t-test).

Further analysis of the DRG neurons responding to αβMe-ATP showed that the percentage of DRG neurons responding to αβMe-ATP (30μM) was significantly reduced in TRPV1$^{-/-}$ (KO) mice compared to TRPV1$^{+/+}$ WT (**Fig 4.7.13**) (59.4±4.9 Vs 36.3±4.1%, $p \leq 0.01$, N=12, unpaired t-test). The total number of responding Vs non-responding cells was 80/132 (WT) Vs 80/234 (KO).

These results suggest there is a decreased overall response to purinergic receptor activation in TRPV1$^{-/-}$ (KO) mice when compared to TRPV1$^{+/+}$ WT through a significant decrease in the total number of DRG neurons activated by αβMe-ATP.

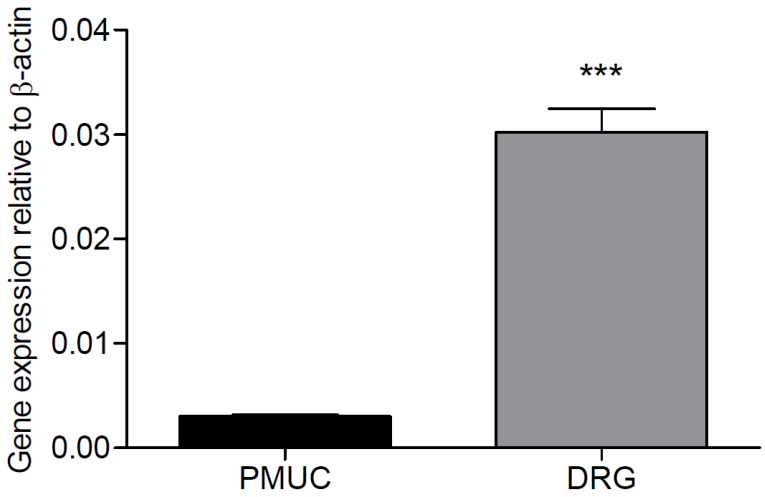

Fig 4.7.1 *Mean±(SEM) expression of TRPV1 receptor mRNA relative to β-actin (ΔCT/CT) in PMUCs and DRG neurons (n=3) (***p≤0.001, n=3).*

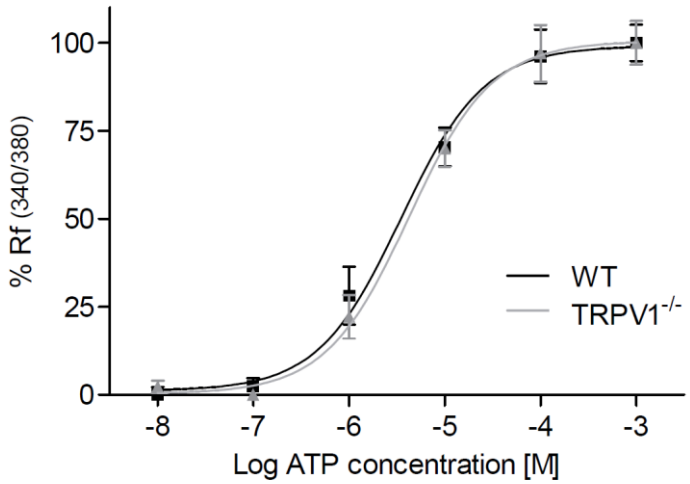

Fig 4.7.2 *Mean±(SEM) concentration response curve of PMUC intracellular calcium to ATP (10nM-1mM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (*N=6, n=360, 470*). Data normalised to % of maximal response.*

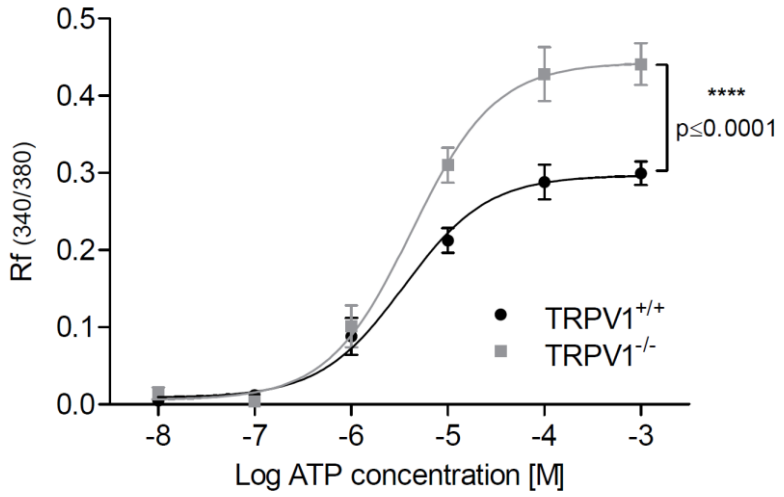

Fig 4.7.3 *Mean±(SEM) concentration response curve of PMUC intracellular calcium to ATP (10nM-1mM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (****p≤0.0001,* N=6, n=360, 470*).*

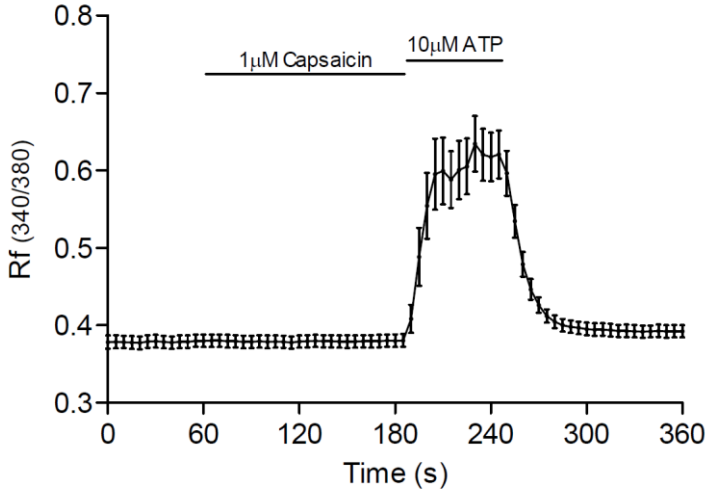

Fig 4.7.4 *Mean±(SEM) change in intracellular calcium levels of PMUCs to capsaicin (1μM) and ATP (10μM)* (N=3, n=57).

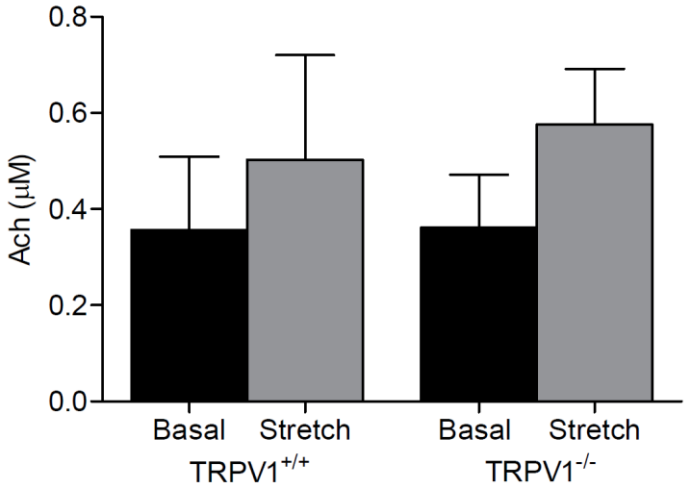

Fig 4.7.5 *Mean±(SEM) intraluminal acetylcholine release from bladders at rest and at a maximum distension of 40mmHg with saline (0.9%) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (n=6).*

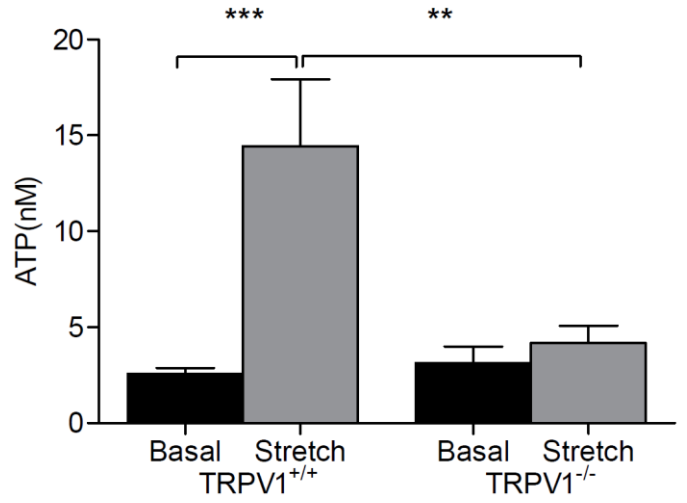

Fig 4.7.6 *Mean±(SEM) intraluminal ATP release from bladders at rest and at a maximum distension of 40mmHg with saline (0.9%) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (n=6).*

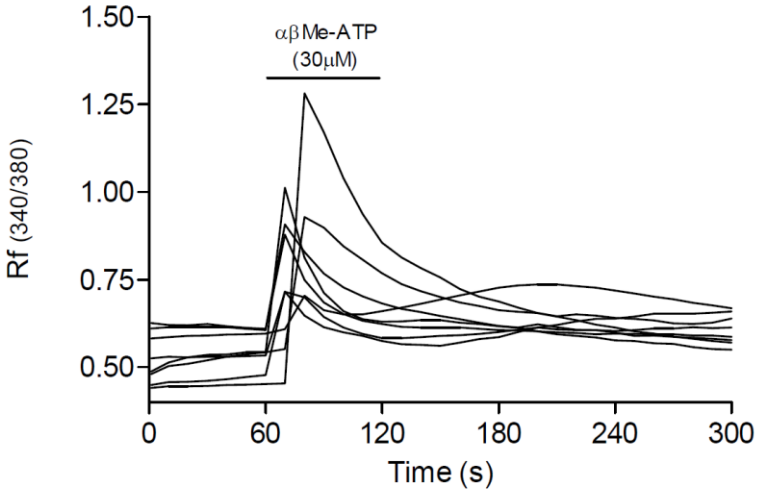

Fig 4.7.7 *Experimental trace showing the variability in individual DRG neurons intracellular calcium responses to αβMe-ATP (30μM) from a single experiment in TRPV1$^{+/+}$ (WT) mice (n=8).*

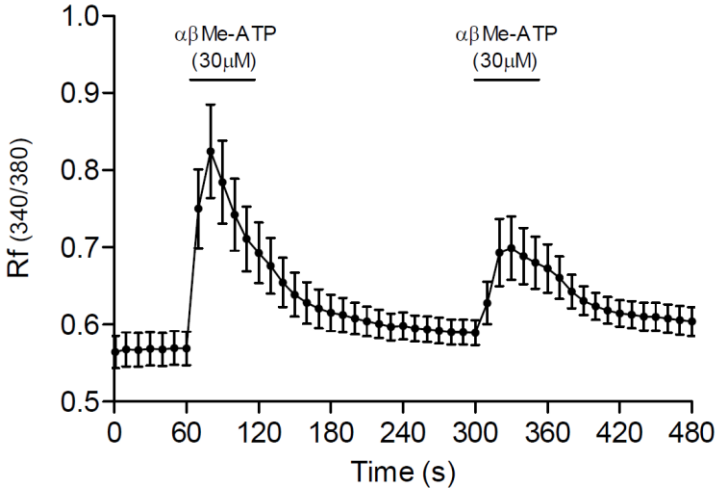

Fig 4.7.8 *Mean±(SEM) time course of intracellular calcium response of DRG neurons to repeat applications of αβMe-ATP (30μM) with 3 minute washout (*N=4, n=32).

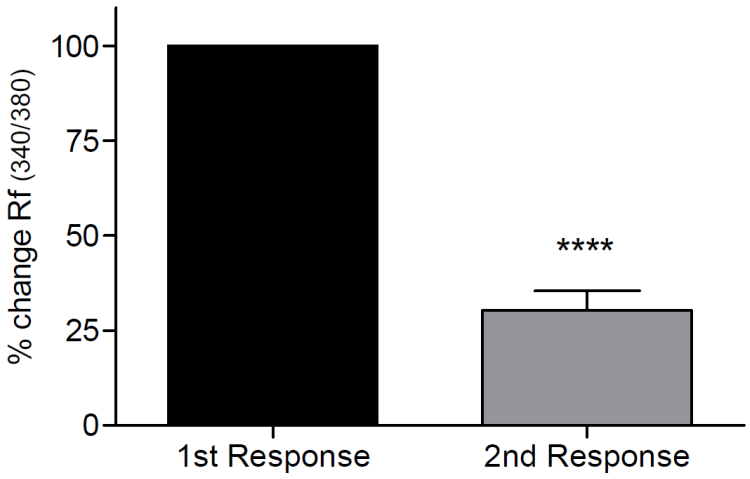

Fig 4.7.9 *Mean±(SEM) percentage change in DRG neuron intracellular calcium in response to repeat applications of αβMe-ATP (30µM) with 3 minute washout in TRPV1$^{+/+}$ (WT) mice (****p≤0.0001, N=4, n=32).*

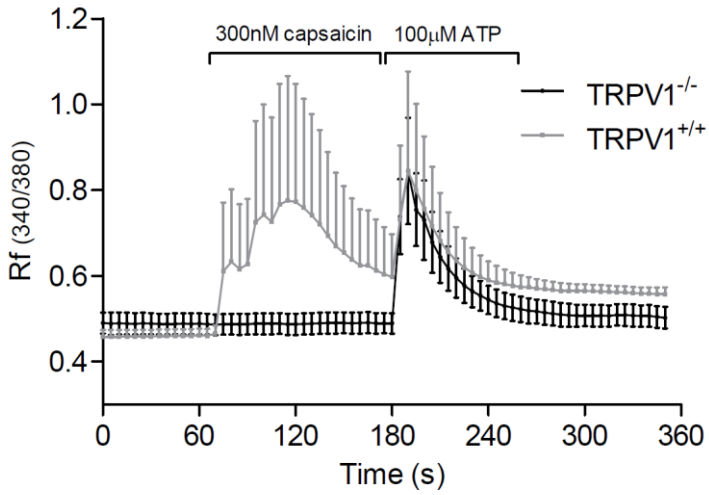

Fig 4.7.10 *Mean±(SEM) time course of intracellular calcium response of DRG neurons to capsaicin (300nM) and ATP (100µM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice* (N=3, n=18).

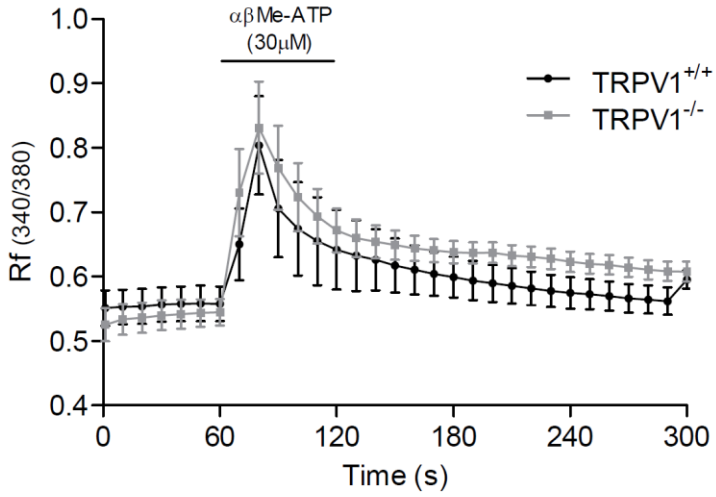

Fig 4.7.11 *Mean±(SEM) percentage change in DRG neuron intracellular calcium in response to αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (N=12, n=80).*

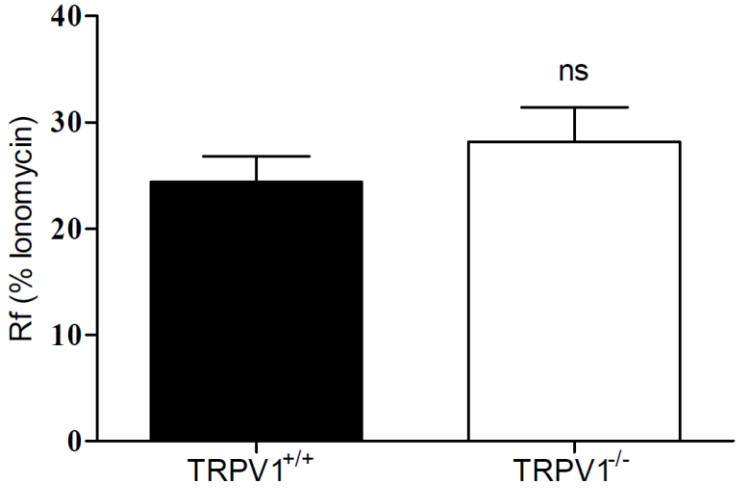

Fig 4.7.12 *Mean±(SEM) peak increase in intracellular calcium of DRG neurons following application of αβMe-ATP (30μM) in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (N=12, n=80).*

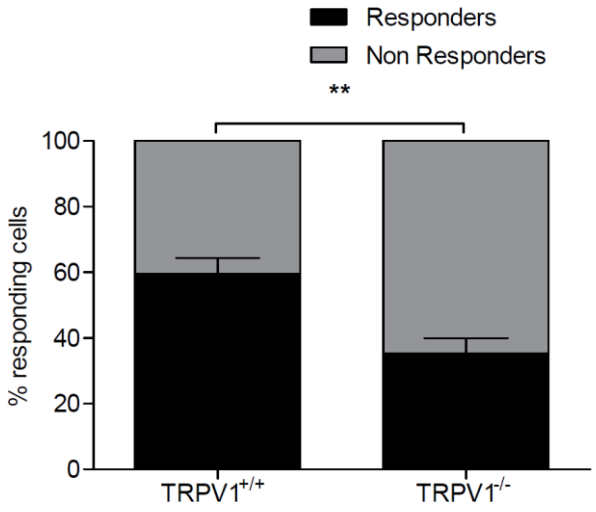

Fig 4.7.13 *Mean±(SEM) percentage of DRG neurons responding to αβMe-ATP (30μM) per experiment from TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice (**p≤0.01, N=12, n=80)*

4.8 DISCUSSION

How does TRPV1 affect normal bladder responses to distension?

As the bladder fills, an increase in afferent nerve discharge is observed with a concurrent increase in intravesical pressure. The initial increase in pressure is accompanied by a sharp increase in afferent nerve activity, which plateaus once the pressure exceeds the physiological voiding threshold.

The first observation obtained in experiments comparing TRPV1$^{-/-}$ (KO) and TRPV1$^{+/+}$ (WT) mice was a significant decrease in the afferent nerve response to ramp distension in KO animals. Importantly, this was not accompanied by a change in detrusor muscle compliance. There is experimental evidence to support a role of TRPV1 as a direct mechanotransducer of bladder afferent discharge generated using a variety of experimental approaches and different animal models, yet no consensus has been reached on how this is achieved mechanistically.

There are many reports supporting a role of TRPV1 as a mediator of reflex bladder activity. Using an anesthetised rat model, Cefalu et al (2009) showed that intravenous administration of a novel TRPV1 antagonist increased micturition threshold volume. Reflex voiding during bladder filling under anaesthesia and c-fos spinal signalling were reduced in TRPV1$^{-/-}$ (KO) mice (Birder, Nakamura et al. 2002), supporting a role for TRPV1 in the afferent input to micturition pathways. Cystometrograms obtained during anesthesia showed more frequent non-voiding contractions, and large amplitude voiding contractions were completely absent in 45% of TRPV1$^{-/-}$ (KO) mice (Birder, Nakamura et al. 2002). Charrua et al (2009) showed that reflex bladder activity in anaesthetised rats and mice was transiently suppressed following duodenal administration of a TRPV1 antagonist. Further, in excised rat bladders capsazepine applied to the serosal side decreased autonomous contractile activity in response to filling (Gevaert, Vandepitte et al. 2007) suggesting a direct role of TRPV1 without connection to spinal reflex mechanisms. These results support a role for TRPV1 as a mediator of detrusor contraction but fail to provide conclusive evidence for an afferent role during distension. Evidence for this comes from the reduction in c-fos signalling during bladder filling in TRPV1$^{-/-}$ (KO) mice (Birder, Nakamura et al. 2002). However, it has since been shown that the contribution of capsaicin sensitive C-fibers to distension may be unmasked during anaesthesia (Cheng and de Groat 2004) and thus these results must be interpreted with care. TRPV1 knockout or the central application of TRPV1 antagonists in many of these studies also limits the emphasis that can be placed on peripheral mechanosensory actions, as TRPV1 is known to be present in central processes. It has also been shown that cutaneous mechanosensation is apparently normal in TRPV1$^{-/-}$ (KO)

mice (Caterina, Leffler et al. 2000) and therefore a direct mechanotransduction role of TRPV1 afferents and stretch/tension detection in the muscle seems unlikely. Furthermore, studies show that knockout of the TRPV1 receptor has no effect on bladder contractions (Charrua, Cruz et al. 2007), or bladder capacity and voiding pressure (Wang, Wang et al. 2008) in anaesthetised mice. This was also shown to be the case in anesthetised rats (Dinis, Charrua et al. 2004) with no change in reflex activity in normal bladders with capsazepine administration.

The most compelling evidence for a role of TRPV1 in mechanosensation comes from the work of Daly et al 2007. In a similar preparation to the one described here, it was shown that bath application of the TRPV1 antagonist capsazepine caused a significant attenuation of afferent discharge in $TRPV1^{+/+}$ (WT) mice and that afferent responses to ramp distension were similiarly attenuated in $TRPV1^{-/-}$ (KO) mice compared to WT, without a change in bladder compliance. Thus, there is a consensus that the TRPV1 receptor is involved in mechanosensation within the mouse bladder. However, as mentioned above and in reference to the physiology of the bladder wall, there are a number of proposed sites for this action, including direct influences on afferent nerve sensitivity, detrusor smooth muscle contractility, and urothelial mediator release. The results of this chapter have focussed on elucidating the mechanisms of this interaction.

It is also important to note that although the present data show a significant change in mechanosensation with knockout of the TRPV1 receptor, there is still a major component of afferent firing during distension that remains. This is consistent with previous cystometric studies showing that reflex responses to bladder distention were attenuated as opposed to abolished in $TRPV1^{-/-}$ (KO) mice and in WT mice treated with TRPV1 antagonists (Birder, Nakamura et al. 2002; Cefalu, Guillon et al. 2009; Charrua, Cruz et al. 2009). There are a number of different mechanisms contributing to the activation of bladder afferent nerves, and tension/contraction of the detrusor smooth muscle is considered a major component of mechanosensation. However, the experiments in this thesis and those by Daly et al (2007) provide firm evidence that the TRPV1 receptor is involved in bladder mechanotransduction via afferent nerves, and that this is not a result of changing bladder compliance. A theory arising from this thesis is that P2X receptors on sensory afferent nerves responding to urothelial ATP released during distension, interact with TRPV1 as a key component of mechanosensation. The hypothesis that TRPV1 may be able to mediate afferent excitability to P2X stimulation through coexpression of TRPV1 and P2X on sensory afferents has been further expored.

Does TRPV1 mediate P2X evoked afferent activity?

There is convincing evidence to suggest that $P2X_3$ sensitive afferent nerves are essential to normal bladder function. There is also evidence that $P2X_3$ receptors co-localise with TRPV1 on bladder afferent nerves (Dang, Bielefeldt et al. 2005), and there are also reports which support an interaction between purinergic receptors and TRPV1 on DRG sensory neurons (Piper and Docherty 2000; Stanchev, Blosa et al. 2009). The aim of this section is to discuss the interactions between TRPV1 and P2X within mouse bladder afferent nerves and determine the location of this interaction.

To investigate further the hypothesis that TRPV1 may mediate afferent sensitivity to P2X receptors, contraction and afferent nerve responses to the P2X receptor agonist αβMe-ATP were examined in $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice, with or without the TRPV1 antagonist capsazepine. The results showed that bladder afferent nerves responded to αβMe-ATP in a concentration-dependent manner with an increase in afferent nerve activity and a concurrent contraction of the detrusor smooth muscle. It was observed, that in the presence of the TRPV1 antagonist capsazepine, and in $TRPV1^{-/-}$ (KO) mice, the afferent response to αβMe-ATP was significantly reduced. It could be proposed based on these results, that the sensitivity of P2X receptors on afferent nerves is modulated by TRPV1, and that the decrease in mechanosensitivity seen during ramp distensions in $TRPV1^{-/-}$ (KO) mice was the consequence of a decrease in afferent sensitivity to urothelial ATP acting through P2X receptors.

In support of this hypothesis, there have been a number of studies implicating the importance of P2X receptors on sensory afferent neurons of the bladder. Vlaskovska et al (2001) identified abundant $P2X_3$ immunoreactive nerve fibers in the sub-urothelial plexus. Further studies have shown that DRG neurons from $P2X_{2/3}$ ($Dbl^{-/-}$ KO) mice have minimal to no response to ATP (Cockayne, Dunn et al. 2005) and DRG neurons from $P2X3^{-/-}$ (KO) mice no longer responded to αβMe-ATP (Zhong, Dunn et al. 2001), indicating that purinergic responses in sensory neurons are largely mediated by $P2X_2$ and $P2X_3$ receptors. $P2X_2^{-/-}$, $P2X_3^{-/-}$, and $P2X_2/P2X_3$ ($Dbl^{-/-}$ KO) mice were also shown to have reduced urinary bladder reflexes and decreased pelvic afferent nerve activity in response to bladder distension (Vlaskovska, Kasakov et al. 2001; Cockayne, Dunn et al. 2005). Furthermore, in WT mice, the P2X receptor antagonists TNP-ATP and PPADS attenuated distension-induced afferent firing (Vlaskovska, Kasakov et al. 2001) whilst αβMe-ATP was seen to potentiate the afferent nerve reponse to distension via both low and high threshold fibers (Rong, Spyer et al. 2002). Similar experiments performed in an in-vitro rat preparation have shown that intravesical administration of αβMe-ATP significantly increased afferent firing which was blocked by TNP-ATP (Yu and de Groat 2008).

Evidence linking TRPV1 and P2X comes from observations that not only do P2X immunoreactive nerves convey sensory afferent information, but that they are co-localised on afferents expressing TRPV1 (Piper and Docherty 2000; Dang, Bielefeldt et al. 2005). Furthermore, co-immunoprecipitation studies have demonstrated the physical association of TRPV1 and $P2X_3$ receptors on DRG neurons (Stanchev, Blosa et al. 2009). It is also interesting that $P2X_3$ immunoreactivity on bladder projecting neurons is significantly increased in patients with neurogenic detrusor overactivity (Brady, Apostolidis et al. 2004) and is significantly reduced in those patients who responded positively to intravesical resiniferatoxin, further suggesting there is functional colocalisation between the TRPV1 and $P2X_3$ receptors on bladder afferent neurons (Brady, Apostolidis et al. 2004). Vlaskovska et al (2001) also showed that 10 minutes after a high dose of capsaicin, the afferent nerve response to bladder distension was signifcantly attenuated, further supporting this hypothesis.

Whilst the investigations described above indicate that there has been interest in a link between TRPV1 and P2X receptors in mediating sensory signalling, the results in this thesis provide the first direct evidence that the TRPV1 receptor is able to influence bladder afferent nerve responses to P2X stimulation, a process considered essential to mechanotransduction during bladder distension. It is also possible from these results to hypothesise that the enhancement in detrusor overactivity symptoms associated with intravesical resiniferatoxin and capsaicin are not only due to desensitisation of TRPV1 but a consequence of decreased afferent nerve responses to urothelial ATP.

In order to draw firm conclusions regarding the ability of the TRPV1 receptor to mediate afferent nerve responses to αβMe-ATP, it was first necessary to investigate the role of TRPV1 in αβMe-ATP induced detrusor contractions as tension in the bladder wall is a major stimulus for afferent firing.

Is the TRPV1 effect due to a change in detrusor smooth muscle contraction?

As briefly discussed above, another consideration within these experiments addresses the hypothesis that TRPV1 is able to modulate detrusor smooth muscle contraction and that there is an interaction between TRPV1 and a specific P2X receptor at the level of the detrusor. Results from this study showed that application of αβMe-ATP induces significant afferent nerve activity as well as a concurrent contraction in detrusor smooth muscle. Furthermore, it was shown that when muscle contraction was blocked by incubation in a calcium free external solution, a significant component of the afferent nerve response to αβMe-ATP remains, albeit small.

Two hypotheses can be proposed to explain these results and they are hard to separate. The first is that there is a component of the αβMe-ATP afferent response that is secondary to muscle contraction and thus removal of detrusor contraction reduces the overall afferent response to αβMe-ATP. The second is that by eliminating calcium from the external solution, it reduces the overall excitability of the afferent neurons. The effects of P2X stimulation are mediated by the influx of cations, predominantly calcium, which results in membrane depolarisation of the afferent terminal and generation of an action potential. A loss of extracellular calcium would decrease the net cation influx during P2X receptor activation and thus the excitability of the neurons.

In support for the first of these hypotheses, contraction of the bladder is a well established mediator of afferent nerve discharge, and a number of mechanoreceptor subtypes have been identified (Zagorodnyuk, Costa et al. 2006; Xu and Gebhart 2008; Zagorodnyuk, Brookes et al. 2009). Therefore the next critical question within this study was to assess if the changes in afferent nerve discharge in $TRPV1^{-/-}$ (KO) mice were a result of changes in detrusor muscle contractability. As the bladder is filled with saline there is an increase in intravesical pressure as the bladder accommodates the increase in volume, and this relationship is referred to as bladder compliance. In experiments with $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice the compliance curves were superimposable, suggesting that the change observed in mechanosensitivity is not a result of altered muscle function. The results obtained in this thesis mirror those found by Daly et al (2007), and support the hypothesis that TRPV1 is involved in mechanosensation and afferent nerve discharge from the bladder during ramp distension rather than an action on the detrusor muscle. This was further tested during extraluminal applications of αβMe-ATP which stimulated a concurrent detrusor contraction with an increase in afferent nerve activity. It was found that neither $TRPV1^{-/-}$ (KO) nor pharmacological blockade with capsazepine affected the detrusor contractions, yet afferent responses were attenuated. Thus, more evidence is provided for a sensory afferent mediated role of TRPV1 in mechanosensation. Further supporting evidence was the continued ability of capsazepine to attenuate the αβMe-ATP induced afferent nerve activity in a calcium free krebs solution in which contractile responses were blocked. The contribution of detrusor contraction to the TRPV1-mediating modulation of both afferent responses to ramp distension and afferent nerve responses to αβMe-ATP can thus be excluded.

There have been previous reports that genetic deletion or pharmacological blockade of the TRPV1 receptor alters bladder contraction dynamics and more importantly in relation to mechanosensitivty, bladder capacity (Birder, Nakamura et al. 2002; Charrua, Cruz et al. 2009) but others have seen no difference in reflex bladder activity, (Dinis, Charrua et al. 2004; Charrua, Cruz et al. 2007; Wang, Wang et al. 2008). It is important, however, to establish that capacity in these studies is defined as

the volume necessary to evoke a micturition response and thus is influenced by reflex mechanisms, particularly in the anaesthetised state. These effects of increased reflex activity could be due to a dampening down of TRPV1 mediated afferent inputs to inhibitory mechanisms arising from the spinal cord that control micturition.

There is a significant effect of both pharmacological blockade and genetic deletion of the TRPV1 receptor on the afferent responses to αβMe-ATP. The results presented here are consistent with a model that excludes a role for detrusor smooth muscle and is most likely due to interactions between TRPV1 and P2X that are co-expressed on sensory afferent neurons innervating the bladder.

Is the TRPV1 effect due to a change in overall afferent excitability?

A previous study into the involvement of TRPV1 in intestinal mechanosensitivity showed reductions in afferent nerve responses to distention (Rong, Hillsley et al. 2004) and concluded that TRPV1 may pre-tune the excitability of these afferents. If this were the case in the bladder, afferent sensitivity to various stimuli would be attenuated and a general, rather than specific mechanosensory role for TRPV1 would be concluded. In order to test this theory, the nicotinic ionotropic receptor agonist DMPP was applied to $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mouse bladders and the afferent response was examined and shown to be unaffected. This provides evidence towards a specific functional link between the TRPV1 and P2X receptors on bladder afferent nerves mediating responses to distension and not a general change in excitability. There is functional evidence for TRPV1 and P2X interaction occuring in rat DRG neurons, where calcium dependent cross-desensitization of the ATP response was observed following prior desensitisation of TRPV1 with capsaicin application (Piper and Docherty 2000). The $P2X_3$ receptor agonist αβMe-ATP also induced less current in the presence of the TRPV1 agonist capsaicin than control (Stanchev, Blosa et al. 2009). However, in this study capsaicin and αβMe-ATP were administered simultaneously and thus controlled for any potential prior desensitisation of the TRPV1 receptor. These studies were not aimed at elucidating bladder signalling and thus the DRG neurons were from throughout the thoracic and lumbar regions.

In support of a subset of afferent neurons showing reduced responses to $P2X_3$ stimulation, Daly et al (2007) found that low threshold afferent activity was exclusively attenuated in $TRPV1^{-/-}$ (KO) mice, in contrast to the generalised view that capsaicin sensitive afferents are nociceptors. It is generally considered that CSPANS are C-fibers responsible for the transmission of noxious stimuli rather than physiological signalling. However, it has been shown in rats that there is a larger population of unmyelinated C-fiber than Aδ-fiber afferents innervating the bladder of rodents (Vera and Nadelhaft

1990; Andersson 2002), and that these fibers respond to slow distension (Morrison 1999) as well as at low bladder pressures (Fowler, Griffiths et al. 2008). It has also been shown in mice that these low threshold mechanosensitive afferents respond to chemical stimuli (Xu and Gebhart 2008), a common characteristic of C-fibers.

Thus, it is proposed that there is an interaction between the $P2X_3$ and TRPV1 receptors at the level of the afferent nerves, and this effect is essential in mediating the afferent nerve responses to ATP released from the urothelium during physiological bladder distension.

Examining individual DRG responses to αβMe-ATP

In order to further test the theory of an afferent mediated role in TRPV1 responses to αβMe-ATP in mechanosensation, the responses of DRG neurons from lumbosacral (L6/S2) and thoracolumbar (T11/L2) regions were examined in $TRPV1^{+/+}$ (WT) and $TRPV1^{-/-}$ (KO) mice.

DRG neurons respond to αβMe-ATP with an increase in intracellular calcium which returns rapidly to baseline over 2-3 minutes. This is consistent with receptor desensitisation because a repeat dose of αβMe-ATP three minutes after washout induces a significantly attenuated response. DRG neurons from WT mice respond to capsaicin with an increase in intracellular calcium, whilst those from $TRPV1^{-/-}$ (KO) mice failed to respond to capsaicin, confirming knockout of the functional receptor. Unexpectedly, in DRG neurons from $TRPV1^{-/-}$ (KO) mice which responded to αβMe-ATP with an increase in intracellular calcium, an equaivalent magnitude of response was seen to that of $TRPV1^{+/+}$ (WT) mice. This argues against the hypothesis that TRPV1 is responsible for setting the excitability of afferents as proposed for afferents supplying the jejunum (Rong, Hillsley et al. 2004). However, while the magnitude of the calcium signal was not different, it was observed that the percentage of DRG neurons responding to the purinergic agonist αβMe-ATP was significantly reduced in the $TRPV1^{-/-}$ (KO). Since receptor expression of a whole host of receptors is developmentally regulated this might suggest a role for TRPV1 in determining P2X receptor expression. Alternatively, it could be proposed that removal of TRPV1 reduces the sensitivity of the $P2X_3$ receptor upon the afferent terminal to respond to purinergic agonism. Thus, it is possible that the responding cells are those which express P2X receptors without TRPV1, and the non-responding proportion of DRGs are those which would normally coexpress TRPV1 and $P2X_3$, and the lack of functional TRPV1 receptor in these neurons renders the $P2X_3$ receptor inactive.

The percentage of a mixed population of thoracolumbar and lumbsacral DRGs responding to αβe-ATP in this study is consitent with other reports (Cockayne, Hamilton et al. 2000; Zhong, Dunn et al. 2001; Dang, Bielefeldt et al. 2005). As with all cell culture experiments the conditions in which the cells are maintained prior to experimentation contributes greatly to the overall effect. This is reflected in the variation observed in the percentage of DRGs responding to capsaicin which ranges from 50% - 90% (Ueno, Tsuda et al. 1999; Yoshimura, Seki et al. 2003; Dang, Bielefeldt et al. 2005). Amongst these capsaicin sensitive neurons there is a distinct population which also respond to αβMe-ATP which ranges from 48 - 76% (Ueno, Tsuda et al. 1999; Dang, Bielefeldt et al. 2005) depending upon the location of the DRGs. Such relative proportions are consistent with the 40% reduction in αβMe-ATP responsive DRGs following TRPV1 knockout seen in this thesis. These results also correlate with the results discussed above showing that $TRPV1^{-/-}$ (KO) mice have a reduced, but not abolished afferent nerve response to αβMe-ATP in the whole bladder. Although a direct mechanism for a reduction in afferent firing following $TRPV1^{-/-}$ (KO) has not been explored in this thesis, there are a number of intriguing possibilities. TRP channels have been recently shown to modulate the sensitivity of other ion channels (Harrington, Hughes et al. 2011), in this case, other TRP channels. This is most likely through extensive phosphorylation of intracellular signalling molecules, which in the case of $P2X_3$ has been shown to be regulated by PKC (Giniatullin, Nistri et al. 2008), calcineurin (King, Chen et al. 1997) and PiP_2 (Mo, Bernier et al. 2009; Mo, Peleshok et al. 2013). Thus it is possible that removal or blockade of the TRPV1 receptor modifies intracellular signalling and reduces the sensitivity of $P2X_3$ receptors.

These results when taken together provide evidence for a role of both P2X and TRPV1 receptors in mechanosensation within the bladder via the release of ATP from the urothelium and actions on sub-urothelial afferent nerves. The precise interactions that occur are still uncertain but the evidence presented here suggests that there is certainly an interaction at the level of the sensory afferent nerve terminal which could underlie the effectiveness of intravesical vanilloid treatment.

Does the urothelium contribute to alterations in mechanosensitivity?

Another potential mechanism by which knockout of the TRPV1 receptor is able to attenuate afferent nerve responses during ramp distension is via actions upon the urothelium. The reduced mechanosensitivity in the bladder seen in the $TRPV1^{-/-}$ (KO) mice could be a downstream effect of a reduction in the release of urothelial factors.

The urothelium is considered to be a sensory epithelium, as it contains many receptors/ion channels including nicotinic, muscarinic, bradykinin, P2X, P2Y and adenosine receptors (Lee, Bardini et al. 2000; Chess-Williams 2002; Chopra, Barrick et al. 2005; Yu, Zacharia et al. 2006; Zarghooni, Wunsch et al. 2007; Chopra, Gever et al. 2008), as well as a number of TRP channels (Birder, Kanai et al. 2001; Stein, Santos et al. 2004; Everaerts, Vriens et al. 2010). In response to mechanical stimulation, the urothelium has the ability to release a number of neurotransmitters and peptides including ATP, acetylcholine, nitric oxide, prostaglandins (Birder 2010) which are reported to act in both a paracrine manner on the underlying interstitial cells and sub-urothelial nerves, but also in an autocrine manner. ATP, due its release by distension and actions on bladder sensory nerves (Vlaskovska, Kasakov et al. 2001) has become the focus of considerable investigations. Augmented release of urothelial ATP has recently been implicated in overactive bladder and has been proposed as a biomarker for this syndrome (Cheng, Mansfield et al. 2013; Silva-Ramos, Silva et al. 2013). If this is the case, then one might hypothesise that a decrease in urothelial ATP might impair sensory function and decrease bladder afferent nerve discharge. This was examined as a possible explanation for the alterations in bladder mechanosensitivity observed in TRPV1$^{-/-}$ (KO) mice.

In TRPV1$^{+/+}$ (WT) mice there was a basal release of ATP from an undistended bladder and a significant increase in ATP release during bladder distension. In TRPV1$^{-/-}$ (KO) mice there was no change in basal release of ATP from control but a significant attenuation of distension evoked ATP release. These results agree with those of others who have previously shown that ATP release is significantly reduced in both excised bladder strips and urothelial cells from TRPV1$^{-/-}$ (KO) mice (Birder, Nakamura et al. 2002). They also went further in demonstrating that capsaicin was able to induce ATP release, an effect blocked by prior incubation with capzasepine. Similar experiments have been performed in rat and rabbit, and capsaicin acting through TRPV1 was shown to induce ATP release (Sadananda, Shang et al. 2009; Dunning-Davies, Fry et al. 2013). The identification of TRPV1 mRNA within the urothelium in this study, albeit in much reduced amounts compared to in DRG neurons, shows that urothelial cells have the ability to synthesise the TRPV1 receptor. The exact mechanism determining ATP release however is unclear, and the basis for TRPV1 modulation of urothelial ATP release is not known. It is important to note that acetylcholine release was not effected by TRPV1$^{-/-}$ (KO) indicating urothelial release of ATP and acetylcholine are mediated via different mechanisms. Indeed, it has been shown that ATP release is dependent on vesicular mechanisms (Knight, Bodin et al. 2002; Birder, Barrick et al. 2003; Collins, Daly et al. 2013) whilst acetylcholine is not (Hanna-Mitchell, Beckel et al. 2007). It was also found that in TRPV1$^{-/-}$ (KO) mice that there is no change in membrane capacitance during stretch (Birder, Nakamura et al. 2002), suggesting that the actions of TRPV1 on ATP release may be up-stream of vesicular exocytosis. As

previously explained, the evidence for TRPV1 as a direct mechanosensor is limited, and accordingly its role in urothelial signalling is not clear. It has been proposed that the TRPV1 channel could form a heteromeric assembly with the mechanosensitive TRPV4 channel (Everaerts, Vriens et al. 2010) similar to that observed in vivo with TRPC channels (Schaefer 2005). However, TRPV1 and TRPV4 channels have been shown to preferentially assemble into homomeric complexes and do not combine by chance (Hellwig, Albrecht et al. 2005; Schaefer 2005). This however, does not rule out the possibility that TRPV1 might interact with other ion channels or non-related proteins to either influence the exocytotic pathways or the function of other mechanosensitive channels within the urothelium.

Indeed, it has previously been shown that antagonism of the TRPV1 receptor is able to inhibit nitric oxide release from the bladder (Birder, Kanai et al. 2001; Birder, Nakamura et al. 2002). The importance of these results in respect to this study are limited as nitric oxide has been reported to either have no role in afferent nerve responses in healthy bladder (Yu and de Groat 2008), or is shown to decrease afferent nerve activity (Caremel, Oger-Roussel et al. 2010; Aizawa, Igawa et al. 2011). Thus, if there was inhibition of nitric oxide release from the bladder urothelium following knockout of the TRPV1 receptor, an increase in afferent nerve responses to distension would be observed rather than the decrease seen in the current study. It is possible that this pathway compensates for a proportion of the functional deficit observed with the abolishment in urothelial ATP following TRPV1 knockout, and therefore masks a more significant decrease in the afferent nerve response to distension. However, without further experiments, this cannot be confirmed.

Despite uncertainty behind the mechanism underlying reduced urothelial release of ATP in the TRPV1$^{-/-}$ (KO) mouse, it is a consistent finding in both rat and mice and from studies in different laboratories. This would imply a role for urothelial ATP within the afferent sensory pathways, and may underlie the positive effects on bladder symptoms seen with the instillation of resiniferatoxin and Botox.

Functional TRPV1 receptors within the urothelium

For TRPV1 to sensitise other components within urothelial cells, the receptor must be coupled to downstream signalling events including phosphorylation pathways that alter the gating properties of various receptors and ion channels including P2X. A rise in intracellular calcium is often the trigger for activating these second messenger cascades and monitoring intracellular calcium for example with Fura2 is an effective way of following activation events because it can be done in real time. In

this respect calcium imaging of PMUCs from TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice allowed direct responses to capsaicin and ATP to be examined. Perfusion of capsaicin onto PMUCs had no effect on intracellular calcium, which is hard to explain given the changes in ATP release observed in the TRPV1$^{-/-}$ (KO) mouse. Nonetheless, this observation is in line with others who have failed to show functional responses to TRPV1 in both isolated mouse and guinea pig urothelial cells (Xu, Gordon et al. 2009; Yamada, Ugawa et al. 2009; Everaerts, Vriens et al. 2010). It is interesting that studies in which functional responses were absent correlated with low expression of mRNA for the TRPV1 receptor. In contrast, other studies performed in rats have shown functional TRPV1 receptors on urothelial cells (Birder, Kanai et al. 2001; Kullmann, Shah et al. 2009) through an increase in intracellular calcium following addition of capsaicin. Species differences could be one explanation. However, another possible explanation may be the length of time the cells are maintained in culture before undertaking the experiments. All studies failing to report TRPV1 evoked responses used the urothelial cells within 48hrs of isolation. Those showing a functional response either do not mention the length of time in culture (Birder, Kanai et al. 2001) or it was over 48hrs (Kullmann, Shah et al. 2009). It is known that culturing primary cells can significantly alter their phenotype. Studies have shown the release of NGF from urothelial cells (Pinto, Lopes et al. 2010; Ochodnický, Michel et al. 2013) and it has been shown that NGF is able to both sensitise and increase membrane expression of TRPV1 (Amaya, Shimosato et al. 2004; Zhang, Huang et al. 2005). Thus, it is tenuous, but possible, that a longer urothelial cell culture provides the environment for the upregulation of TRPV1 receptors that underlies their functional response. This is also a potential mechanism whereby inflammation and/or damage to the urothelial cell layer can induce TRPV1 and a subsequent increase in ATP release which may underlie sensory urgency and pain.

Following observations that urothelial ATP release was attenuated in TRPV1$^{-/-}$ (KO) mice, the response of PMUCs to exogenous ATP was tested using calcium fluorescence. The responses to ATP in TRPV1$^{+/+}$ (WT) and TRPV1$^{-/-}$ (KO) mice had an identical response profile and there was no change in the concentration of ATP required to elicit a maximal response. However, what is of particular intrigue is that the maximum responses to ATP in urothelial cells from TRPV1$^{-/-}$ (KO) mice were significantly higher (≈50%) than the responses from TRPV1$^{+/+}$ (WT) cells. This is the first study to report this augmented response in urothelial cells and requires corroboration. Such augmentation might reflect compensatory changes occurring as a result of the TRPV1 knockout. The urothelial response to ATP, although not well understood, has been shown to induce further ATP release (Sun and Chai 2005) which likely plays a role in the downstream effects of mechanosensation and as such, through removal of TRPV1 and a significant attenuation in ATP release during distension, there is an

upregulation of purinergic receptors to the membrane which underlie the increased response to exogenous ATP.

The TRPV1 receptor may have two synergistic roles in mediating the mechanosensitivity which when knocked out could result in the deficit in mechanosensitivity observed during bladder distension. A decrease in the release of ATP from the urothelium, coupled with a decrease in afferent nerve sensitivity to ATP could combine to result in a decrease in afferent nerve sensitivity. However, it is necessary to mention that the contribution of these pathways to afferent discharge during normal bladder distension is only small. However, the relative density of suburothelial $P2X_3$ and TRPV1 immunoreactive nerves has been shown to increase in disease and thus this pathway may contribute a larger proportion of the mechanosensory response to distension during pathophysiological conditions.

Conclusions

The results in this chapter have provided evidence towards the already comprehensive literature supporting a role for ATP and P2X receptors in mechanosensitivity within the bladder, specifically its actions upon the sensory afferent nerves. The results in this chapter have also presented evidence for a significant role of the TRPV1 receptor in mediating bladder responses to P2X agonists, particularly during ramp distension. The loss of TRPV1 function resulted in decreased afferent nerve responses to ramp distension and following further investigation it was found that genetic deletion of TRPV1 or its pharmacological antagonism resulted in a decreased afferent response to P2X receptor stimulation. This was a specific effect on P2X receptor function and was not due to a loss of general excitability. This leads to the proposal that the interaction between TRPV1 and P2X contributes to mechanosensation within the bladder and that the functional presence of TRPV1 may be essential for the effects of endogenous ATP on sensory signalling to be fully manifested. In addition, a synergistic interaction of TRPV1 and the purinergic system is evident in the urothelium where loss of TRPV1 significantly attenuated stretch evoked ATP release. The present results provide a mechanistic basis for the decrease in sensory symptoms seen following resiniferatoxin and capsaicin treatment for lower urinary tract symptoms, although the relative contribution of the urothelium and the sub-urothelial afferent nerves remains an unknown. Understanding the relative role of TRPV1 in urothelial cells Vs DRG neurons may require cell-specific conditional knockouts based on cre-loxP technology.

CHAPTER 5: THE ROLE OF TACHYKININ RECEPTORS IN MECHANOSENSATION

5.1 INTRODUCTION

Background of Tachykinin receptors

The tachykinin family of peptides consisting primarily of substance P, neurokinin A (NKA), and neurokinin B (NKB) are principally found within the central and peripheral nervous system. All three tachykinins, bind to, and act as full agonists for all three tachykinin receptor subtypes but show a defined order of potency such that substance P binds preferentially to NK1, NKA preferentially binds NK2, and NKB is more selective for NK3 (Mussap, Geraghty et al. 1993; Regoli, Boudon et al. 1994; Maggi and Schwartz 1997).

Tachykinins in the bladder

Within the bladder, tachykinin neuropeptides are largely contained within capsaicin sensitive primary afferent neurons (CSPANS) (Lecci and Maggi 2001; Avelino, Cruz et al. 2002) but it is also possible for tachykinins to be present in capsaicin-insensitive neurons, especially following inflammation or insult (Neumann, Doubell et al. 1996; Hunter, Myers et al. 2000; Carr, Hunter et al. 2002). Activation of CSPANS has been shown to trigger the release of tachykinins, and treatments which desensitise and disrupt the sensory function of CSPANS has been shown to produce positive outcomes in both idiopathic and neurogenic bladder disorders (Brady, Apostolidis et al. 2004; Apostolidis, Gonzales et al. 2006) through an increase in bladder capacity, and decrease in sensations of urgency and frequency. It has been proposed that these beneficial effects are mediated in part by changes in the release of endogenous tachykinins. However, the precise roles that tachykinin receptors play within the bladder, and particularly their role in sensory signalling are yet to be determined.

A dual role for CSPANS in sensory signalling was proposed by Maggi et al (1998), whereby in the process of CSPAN mediated afferent signalling, there is a concurrent release of neuropeptides from the peripheral terminals which leads to an effector response in the periphery as part of the axon reflex. This process was termed a sensory ‘efferent’ function and it is these proposed actions, which have implicated tachykinins in a role in bladder physiology and pathophysiology. Tachykinin containing CSPANS, which are found to innervate the detrusor smooth muscle, but more densely innervate the suburothelium of humans (Keast and de Groat 1992; Smet, Moore et al. 1997) and rats

(Maggi 1993), thus have the potential to influence a number of bladder structures including the detrusor smooth muscle, interstitial cells, urothelium, and sensory afferent nerves.

CSPANS synthesise and release substance P and NKA (Dalsgaard, Haegerstrand et al. 1985; Hökfelt, Pernow et al. 2001) and also express the receptors for these neuropeptides (Brechenmacher, Larmet et al. 1998) whilst more recently, NKA has been shown to enhance the excitability of capsaicin sensitive DRGs (Sculptoreanu and de Groat 2007). These observations have raised the possibility that neurokinins may act in an autofeedback manner to regulate afferent terminal excitability. Indeed, tachykinins have been shown to exert significant effects on the excitability of capsaicin sensitive lumbo-sacral DRG neurons including an increase in calcium currents (Sculptoreanu and de Groat 2003) and an increase in action potential frequency in response to sustained depolarisation currents accompanied by a decrease in action potential threshold (Sculptoreanu and de Groat 2007). There is also an enhancement of capsaicin evoked currents (Sculptoreanu, Aura Kullmann et al. 2008), and it has recently been suggested that these effects are mediated in part due to a positive shift in the activation curve of low threshold potassium-currents (Sculptoreanu, Artim et al. 2009). As mentioned above, nerves immunoreactive for NKA are found in the detrusor muscle (Burcher, E et al. 2000; Andersson 2002) but more densely populate the sub-urothelium, (Smet, P et al. 1997), and thus an autoregulatory mechanism of tachykinins on afferent excitability could have wide ranging effects within the bladder, including the transduction of mechanical stimuli during bladder distension.

Actions on bladder contraction

As described briefly earlier, CSPANS innervating the bladder have been shown to have two functions. In addition to a known sensory function, they also exhibit an ‘efferent’ component composing the release of tachykinins, which can bind to tachykinin receptors on detrusor smooth muscle causing contractions.

There is evidence that endogenous stimuli can induce the release of tachykinins, including muscle stretch (Lecci, Giuliani et al. 1998) which provides physiological relevance for tachykinins during bladder distension. Stimulation of CSPANS with a non-desensitising dose of capsaicin is also known to stimulate the release of tachykinins (Maggi, Patacchini et al. 1991; Lecci, Giuliani et al. 1997), and the resultant contractile response of the detrusor smooth muscle can be blocked by prior administration of joint NK1 and NK2 receptor antagonists (Maggi, Patacchini et al. 1991) or specific NK2 receptor antagonists (Lecci, Giuliani et al. 1997) in the rat.

Both NK1 and NK2 receptors are present on detrusor smooth muscle of a number of species but there is a large degree of difference in the effects. Bladder contractions via NK1 receptor agonists are limited to the rat and guinea-pig (Longmore and Hill 1992) via activation of phospholipase C and IP_3 (Suman-Chauhan, Guard et al. 1990; Martin, Wheeler et al. 1997), which are also activated by NK2 receptors. In many species, NK1 receptors are limited to the smooth muscle and blood vessels of both the detrusor and sub-urothelium (Mussap, Geraghty et al. 1993; Burcher, Zeng et al. 2000).

Within the bladder, the actions of NKA are commonly found on the detrusor smooth muscle. NK2 receptors are present on detrusor smooth muscle of a number of species, including hamster and rabbit (Guard, Pain et al. 1993), guinea pig (Longmore and Hill 1992), sheep (Tucci, Bolle et al. 2001), dog (Rizzo and Hey 2000), pig, and human (Giuliani, Patacchini et al. 1993; Burcher, Zeng et al. 2000; Templeman, Sellers et al. 2003) and have been shown to elicit powerful bladder contractions. NK2 mediated detrusor smooth muscle contraction has been linked to IP_3 accumulation (Suman-Chauhan, Guard et al. 1990; Torrens, Beaujouan et al. 1995; Martin, Wheeler et al. 1997) and rho-kinase has also been implicated in NKA induced detrusor contraction in rats (Wibberley, Chen et al. 2003; Quinn, Collins et al. 2004). As well as rho-kinase and IP_3 accummulation, NKA induced detrusor contraction is dependent on extracellular calcium though both receptor mediated and voltage gated calcium channels (VGCC) (Quinn, Collins et al. 2004) and it has been previously shown that this component of NK2 receptor-mediated bladder contraction largely depends on the activation of nifedipine sensitive calcium channels (Maggi, Giuliani et al. 1989). The role of prostanoids is slightly more contentious; with a significant effect of prostanoids on the L-type calcium channel component of NK2 mediated detrusor contraction in the hamster (Tramontana, Catalioto et al. 2000; Candenas, Lecci et al. 2005), whilst blockade of cycloxygenase failed to inhibit tachykinin receptor-mediated detrusor smooth muscle contractions in rats, guinea-pigs, dogs and humans (Quinn, Collins et al. 2004; Candenas, Lecci et al. 2005).

Thus, it is possible that tachykinin neuropeptides released from CSPANS in response to muscle stretch are able to provide a degree of control to the contraction of the detrusor smooth muscle and could provide an endogenous tone to the bladder wall during bladder distension.

Actions on the urothelium

As described above, the greatest density of tachykinin immunoreactive fibers innervating the bladder is found within the suburothelium (Keast and de Groat 1992; Smet, Moore et al. 1997).

There is the potential therefore that tachykinins released from terminal varicosities within the sub-urothelium are able to influence the underlying urothelium and interstitial cells.

Despite a lack of functional evidence it is considered that the NK2 receptor is also present on the urothelium. Bahadory et al (2013) showed expression of NK2 receptor mRNA on urothelial cells whilst indirect evidence of a functional NK2 receptor upon the urothelium is presented by eradication of an NK2 receptor mediated prostanoid release following urothelial removal (Tramontana, Catalioto et al. 2000) and NK2 receptor induced nitric oxide production in cultured rat urothelial cells (Candenas, Lecci et al. 2005). It has also been shown that intravesical instillation of NKA, but not substance P or NKB into the bladder lumen is able to stimulate micturition in conscious rats (Ishizuka, Mattiasson et al. 1995). The authors of this study concluded that the actions of NKA on the micturition reflex must be secondary to direct actions upon the urothelium, long before the current consensus that the urothelium plays an important role in the control of micturition. Maggi et al (1991) also observed facilitation of reflex micturition by intravesical NKA in anaesthetised rats, coupled with a reduced bladder capacity and residual volume (Maggi, Giuliani et al. 1991). Interestingly, in an in vitro model of guinea pig whole bladder, the instillation of intravesical NKA facilitated the occurrence of rhythmic contractile activity (Maggi, Giuliani et al. 1991) which was not observed with intravesical instillation of NKA in rats (Ishizuka, Mattiasson et al. 1995).

A more recent avenue of research has implicated a novel structure in mediating the actions of NKA in the bladder. Using porcine bladder strips, NKA was found to directly mediate mucosal contractions in the presence of TTX and indomethacin and a role for suburothelial interstitial cells was proposed (Sadananda, Chess-Williams et al. 2008).

Role in the micturition reflex

As described above, intravesical administration of tachykinins has been shown to stimulate the micturition reflex (Maggi, Giuliani et al. 1991; Ishizuka, Mattiasson et al. 1995). A number of other studies using a variety of techniques have also shown tachykinins can influence the micturition reflex, although the role of peripherally mediated tachykinin responses has still not been sufficiently explored

A physiological role for tachykinin receptors in mediating micturition comes from a study in which 83% of mutant mice (lacking the preprotachykinin gene) exhibit urinary retention and overflow incontinence during cystometrograms (Kiss, Yoshiyama et al. 2001). Also, an NKA induced increase in

basal intravesical pressure followed by micturition was observed immediately after intra-arterial NKA (Ishizuka, Mattiasson et al. 1995). Bushfield et al (1995) followed a similar protocol with intravenous administration of NKA in guinea pigs and also elicited bladder contraction, interestingly, this effect was blocked by pretreatment with a desensitising dose of αβMe-ATP and atropine combined with hexamethonium suggesting the involvement of reflex mechanisms, and it was proposed that activation of preganglionic NK2 receptors could potentiate ATP and ACh release from efferent fibers to promote muscle contraction (Bushfield, Metcalfe et al. 1995). Intravenous (IV) administration of an NK2 agonist was shown to stimulate a significant increase in afferent nerve responses to bladder distension without a change in intravesical pressures (Kibble 1996). In separate experiments, an NK2 antagonist administered IV was shown to decrease peak afferent discharge rate and increase the intravesical pressure at which it occurred (Kibble 1996), and it was concluded that greater levels of receptor stretch are necessary to achieve normal levels of afferent nerve discharge in the presence of an NK2 receptor antagonist. There was no mention, however, of volumes required to elicit these pressures and it has previously been shown that the NK_2 receptor antagonist MEN 11420 induces a relaxation of the basal tone of the urinary bladder that is more apparent at high volumes of distension (Lecci, Giuliani et al. 1998).

Studies utilising tachykinin antagonists have explored the role of endogenous tachykinins in the peripheral control of micturition in both physiological and pathophysiological states. It has been found that there is only a minor contribution of NK1 and NK2 receptors in controlling micturition parameters including bladder capacity and the amplitude of micturition contractions during cystometry recordings of normal rats (Lecci, Giuliani et al. 1993; Ishizuka, Igawa et al. 1994; Lecci, Tramontana et al. 1998). The effect of tachykinin antagonists are revealed in models of bladder irritation and outflow obstruction, with a significant role for the NK2 receptor in attenuating increases in micturition reflex parameters back towards physiological levels (Lecci, Giuliani et al. 1993; Ishizuka, Igawa et al. 1994; Lecci, Tramontana et al. 1998).

These results are consistent with a peripherally mediated release of tachykinins mediating bladder overactivity and are consistent with the positive outcomes in both idiopathic and neurogenic bladder disorders following desensitisation of CSPANS (Brady, Apostolidis et al. 2004; Apostolidis, Gonzales et al. 2006). Indeed there is a significant increase in tachykinin immunoreactivity in the sub-urothelium of patients with idiopathic detrusor overactivity (Moore K H 1992; Smet, Moore et al. 1997) and there is a significant increase in tachykinin immunoreactive fibers, which was not as a result of overall increases in nerve density, observed in animal models of chronic inflammation (Callsen-Cencic and Mense 1997).

Role in pathology

A number of bladder symptoms have been linked with alterations in the tachykinin containing structures of the bladder. As mentioned above, changes in tachykinin related sensory neuron innervations have been observed in the sub-urothelium of patients with idiopathic detrusor overactivity (Moore K H 1992; Smet, Moore et al. 1997). Spinal cord injury induced hyperreflexia is significantly reduced by intravenous administration of an NK2 receptor antagonists (Abdel-Gawad, Dion et al. 2001; Abdel-Karim, Barthlow et al. 2005). Atherosclerosis induced ischemia has also been shown to significantly increase the frequency of spontaneous bladder contractions in vivo with a concurrent increase in NK2 receptor expression (Azadzoi, Radisavljevic et al. 2008) whilst electrical field stimulation (EFS)-induced smooth muscle contractions were significantly greater in the ischemic tissues and this was selectively reduced by an NK2 receptor antagonist (Azadzoi, Radisavljevic et al. 2008). In a model of diabetic cystopathy, polypeptide inhibitors of TRPV1 decreased TRPV1 dependent release of tachykinins from bladder afferents (Philyppov, Paduraru et al. 2012). CSPANS are symptom dependently upregulated in interstitial cystitis patients (Liu, Yang et al. 2014) as is NGF (Liu, Yang et al. 2014), which is also seen in OAB patients (Tyagi, Tyagi et al. 2013). There is a body of evidence which suggests that there is increased expression of tachykinins in response to NGF (Girard, Tompkins et al. 2012) and significantly heightened tachykinin CSPAN immunoreactivity following increased VEGF in the sub-urothelium (Saban, Davis et al. 2011), both known to increase during inflammation. Intravesical VEGF was also found to significantly reduce micturition pressure and inter-micturition interval (Malykhina, Lei et al. 2012) which could be related to changes in tachykinin immunoreactivity. In NGF overexpressing mice there was a marked increase in the density of CGRP- and substance P-positive C-fiber sensory afferents in the suburothelial nerve plexus which was accompanied by reduced urinary bladder capacity and increases in the number and amplitude of non-voiding bladder contractions (Schnegelsberg, Sun et al. 2010).

These results provide a link by which inflammation can induce recruitment of CSPANS and an increase in bladder content of tachykinins, which are able to potentiate detrusor contraction at the periphery, and hyperexcitabilty in the spinal cord through autofeedback regulation of afferent terminals.

The role of tachykinin neuropeptides in mediating responses to bladder irritation has also been investigated. The increased c-fos expression seen in spinal neurons following bladder irritation (Birder and de Groat 1992) is significantly attenuated with pretreatment with an NK2 antagonist (Kiss, Yoshiyama et al. 2001). Intravesical antigen infusion in ovalbumin sensitised guinea pigs induced abdominal licking, whilst pre-treatment with a desensitising dose of capsaicin abolished

intravesical antigen infusion behaviour (Ruggieri, Filer-Maerten et al. 2000), and an intra-peritoneal (IP) injection of NK1 and NK2 but not NK3 antagonists prior to infusion of antigen reduced antigen evoked behaviours.

These results suggest there is not only modulation of the micturition reflex and afferent nerve activity by tachykinins in the periphery, but there is also a role for tachykinins in the production of physiological spinal mediated reflex actions and possibly the sensitisation of spinal afferents during spinal insult. Pharmacological research extending back over 25 years has established a complex picture of tachykinins in the bladder acting via multiple receptors and intracellular events in various cell types to initiate and modulate bladder contraction and so influence micturition. More recently, the focus has been on how these mechanisms might be influenced by pathophysiology and so contribute to bladder overactivity in patients with chronic inflammation, cystitis and idiopathic detrusor overactivity. There remain, however, a number of uncertainties in this area of research. The complex interaction between the sensory nerves that are a major source of tachykinins within the bladder, and the cellular environment that signal to, and receive signals from, these sensory nerves which express tachykinin receptors has thus far proved difficult to discern.

In particular there is still very little evidence of a tachykinin role in normal bladder function, and the studies which have been conducted have failed to determine the precise role of tachykinins within the bladder. There has been little experimental differentiation between actions which could be a result of direct sensory afferent nerve input to the micturition pathways or alterations in mechanosensitivity second to urothelial and detrusor function. As such, it is still unknown if alterations in detrusor contraction are the trigger for micturition via release of tachykinins from afferent nerve terminals, or if tachykinins are able to directly act on the afferent nerves to stimulate reflex micturition.

This thesis chapter will offer a new approach to the study of tachykinins within the mouse bladder and aims to address the paucity of recent functional evidence regarding peripheral regulation of tachykinin receptors and assesses the emergence of a sensory role for the urothelium in this process.

5.2 EXPERIMENTAL PROTOCOLS

The main methodology is detailed in chapter 2. Here the emphasis is on the specific protocols that were employed to address the role of tachykinins in mediating bladder muscle contraction, afferent nerve activity, and urothelial receptor function.

5.2.1 Intravesical pressure and afferent nerve recording.

Bladder contraction

In order to measure contractile responses of detrusor smooth muscle and corresponding afferent nerve activity, bladders were perfused intravesically with 0.9% saline to a maximum pressure of 12mmHg. The syringe pump was stopped but the outflow tap remained closed, keeping a set volume within a moderately distended bladder. Distension was maintained to allow time for the bladder muscle to accommodate this volume (**fig 2.4.1**) and become stable. Stock solutions of NKA (10nM-3µM), substance P (1nM-300nM), and the specific NK3 receptor agonist senktide (100pM – 300nM) and αβMe-ATP (30µM) in dH_20 or DMSO were prepared and added as a 900µl aliquot to the experimental bath with a volume of 30ml of krebs buffer. In experiments in which multiple applications of agonist were applied in the same experiment, a washout period of 45minutes between each dose ensured receptors were not desensitised.

NK2 antagonist GR159897 (stock @1mg/ml DMSO) (100nM) and Nifedipine (stock@50mg/ml) (1µM) were diluted in krebs and applied via the perfusion pump (5ml/min) to the experimental bath for 30min prior to agonist application.

Ramp distensions

All ramp distensions in this study were performed at a rate of 30µl/min to a maximal pressure of 30mmHg. Pilot experiments had established that these conditions were optimal to evoke sustained and reproducible detrusor contractions, which were a major focus of this study. As can be observed in **fig 5.2.1,** control distensions of 30µl/min produce reproducible responses to distension.

Intravesical administration of drugs

For intravesical infusion of drugs, either control (0.9% saline + vehicle), NKA (300nM), senktide (300nM) or substance P (300nM) were infused into the bladder lumen via the syringe pump. The syringe pump provided constant infusion of drug or saline at 30µl/min with the outflow tap open to prevent bladder distension and in order to ensure that the full dose of the drug was present in the bladder during the following ramp distension. Agonists were infused immediately following bladder emptying from control distensions and for a minimum of 7 minutes, whilst antagonists were infused intravesically for at least 30minutes prior to, and during the addition of an agonist.

Periods of control or agonist infusion without bladder distension were analysed for spontaneous baseline afferent discharge allowing for the dead space between the syringe pump and the bladder which was 120µl, and a latency of response of 4 minutes.

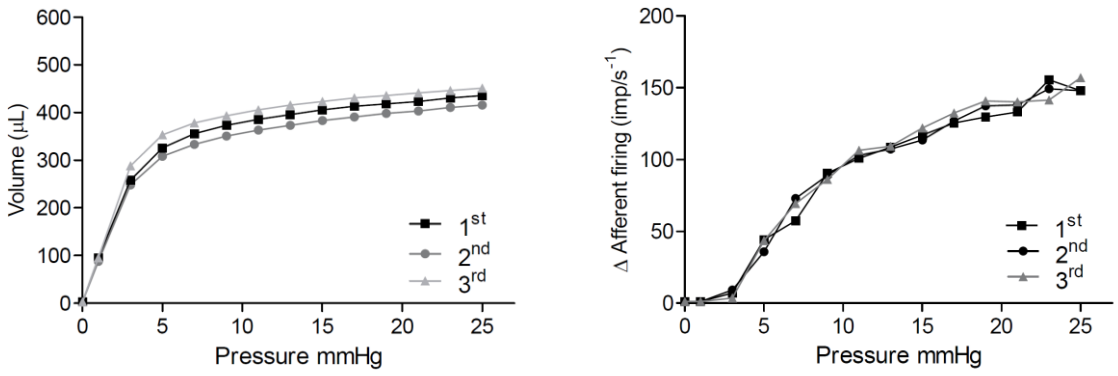

Fig 5.2.1. *Pressure/volume and pressure/nerve responses to three repeat distensions at 30µl/min prior to the start of an experiment. The compliance and afferent response to distension are reproducible.*

Extravesical administration of drugs

Extravesical application of agonists/antagonists for examination of ramp distensions, either control (krebs +vehicle), NKA (300nM), senktide (300nM), substance P (300nM) or GR159897 (100nM) were applied via the perfusion pump to ensure a steady concentration of compound within the experimental bath without the need to stop perfusion during bladder distension.

5.2.2 Mediator release

Mediator release experiments were performed with infusion set at 30µl/min and to a maximal pressure of 30mmHg to replicate the protocol for ramp distensions in this study.

Basal samples of intraluminal contents were taken during a 7 minute infusion of vehicle/agonist @30µl/min with the outflow tap open to prevent bladder distension, the outflow tube was placed into a sterile Eppendorf tube placed on dry-ice to prevent post-hoc degradation of substrates. Immediately following ramp distensions, the syringe pump was stopped and the ending of the outflow tap was placed into a sterile Eppendorf tube placed in dry-ice to collect the intravesical contents whilst the bladder was left to empty passively.

Intravesical contents from basal and distension (stretch) in control and drug treated preparations were assayed for ATP (Molecular Probes® A22066), ACh (Molecular Probes® A12217) and Prostaglandin E_2 (Cayman Chemical® 514010) using commercially available kits. Mediator concentrations in test samples were calculated from a standard curve of known concentrations with blank values subtracted.

5.2.3 qPCR

qPCR experiments were performed as described in chapter 2. PMUCs were isolated from mice and cultured for 24hrs at 37°C. mRNA was harvested, converted to cDNA and RT-qPCR was performed using SYBR-Green fluorescence with the primers listed.

Gene		Sequence '5-3'	Product	Position	Tm	Chromosome
NK1	Forward	GACACTTAGTCTGCCAAGAGC	193bp	3561-3753	58.7	6
	Reverse	CTCCACATGCTGGATAGAGC			57.8	
NK2	Forward	GGATGGTGACATGGCTCAGC	117bp	1879-1995	61.1	10
	Reverse	GGACAGCTTGCAGACGTTGG			61.8	
NK3	Forward	CCTCATTCTCCTAACCATGCC	205bp	1681-1885	58.1	3
	Reverse	CAGACACCGGATACTTAGAGC			57.6	

Table 5.2.1 *Primer sequence, product size, gene loci, and Tm for purinergic receptors investigated in this study.*

5.2.4 Calcium imaging

The calcium imaging protocol is described in more detail in the major methods chapter.

Calcium imaging was performed using an excitation wavelength of 510nm light and ratios were calculated from emissions at 350 and 380nm with background fluorescence deducted. NKA (stock @1mg/ml H_2O) (300nM) and Ionomycin (stock @5mg/ml DMSO) (5µM) were diluted from stock into HEPES solution and applied using a gravity perfusion system at a rate of 1.5ml/min for 5 minutes. Data are represented as a ratio of fluorescent intensity (Rf) (350/380) and also as a percentage of Ionomycin fluorescence. N = number of mice, n = number of cells.

5.3 CHARACTERISATION OF BLADDER RESPONSES TO EXTRALUMINAL TACHYKININ RECEPTOR AGONISTS

In these experiments, the bladder is partially filled to a pressure of 12mmHg (as described in section 5.2) in order to observe a rise in intravesical pressure.

Bath application of the NK2 receptor agonist NKA elicited a concentration-dependent increase in intravesical pressure as a result of detrusor smooth muscle contraction, and a concomitant increase in afferent nerve firing (**fig 5.3.1A**). The contractile response consisted of a tonic increase in baseline pressure upon which phasic contractile activity was superimposed. The increase in afferent firing matched in both magnitude and timecourse the increase in intraluminal pressure. Indeed, on an expanding time base (**fig 5.3.1B**) the phasic increases in pressure and afferent discharge can be seen to be temporally linked.

Bath application of substance P (300nM) and senktide (300nM), which act predominantly via NK1 and NK3 receptors, also stimulated bladder contraction and a corresponding increase in afferent nerve activity but with a much reduced potency to that of NKA (**fig 5.3.2**). NKA (300nM) elicited contraction (6.9±0.7mmHg) and afferent nerve activity (78.1±15.5imp/s^{-1}, n=6) significantly greater than that of sub p (300nM) (1.3±0.7mmHg, 4.0±1imp/s^{-1}, p≤0.001 n=4) and senktide (300nM) (1.5±0.3mmHg, 12±2imp/s^{-1}, p≤0.001, n=4, one-way ANOVA, Bonferroni multiple comparisons post hoc test). The NK2 receptor antagonist GR159897 (100nM) significantly attenuated NKA (300nM) induced detrusor muscle contraction (6.9±0.7 Vs 2.8±0.8mmHg, p≤0.001, n=4) and afferent nerve activity (53.0±11.5 imp/s^{-1},n=4, one-way ANOVA, Bonferroni multiple comparisons post hoc test, **fig 5.3.3**). An example trace showing GR159897 antagonism of NKA induced muscle contraction and afferent activity can be seen in **fig 5.3.4**. Bursts of afferent nerve activity correlated with rhythmic detrusor contractions were partially conserved, but a significant component of the sustained pressure and afferent firing was blocked. Nifedipine (1μM) was also able to significantly attenuate NKA (300nM) induced peak afferent nerve activity (78.1±15.5 Vs 1.3±0.3imp/s^{-1}, p≤0.001, n=6) and detrusor muscle contraction (6.9±0.7 Vs 0.81±0.08mmHg, p≤0.001, n=6, one-way ANOVA, Bonferroni multiple comparisons post-hoc test, **fig 5.3.3**). An experimental trace of NKA induced afferent nerve activity and detrusor smooth muscle contraction prior to, and following, incubation with nifedipine can be seen in **fig 5.3.5**. Nifedipine was able to completely abolish NKA induced nerve and muscle activity. It is evident, however, that baseline afferent activity remained suggesting that spontaneous activity is uncoupled from muscle contraction.

These data demonstrate that detrusor muscle contraction and afferent nerve responses within the mouse bladder are predominantly NK2 mediated and moreover that the detrusor contraction drives the augmentation in afferent discharge. It can be proposed therefore; that the actions of NKA are limited to the detrusor muscle and afferent nerve discharge from the bladder towards the CNS is a consequence of NK2 receptor mediated contraction only. In order to test this hypothesis, the ability of tachykinin agonists to influence detrusor smooth muscle and afferent nerve activity during bladder distension was further investigated.

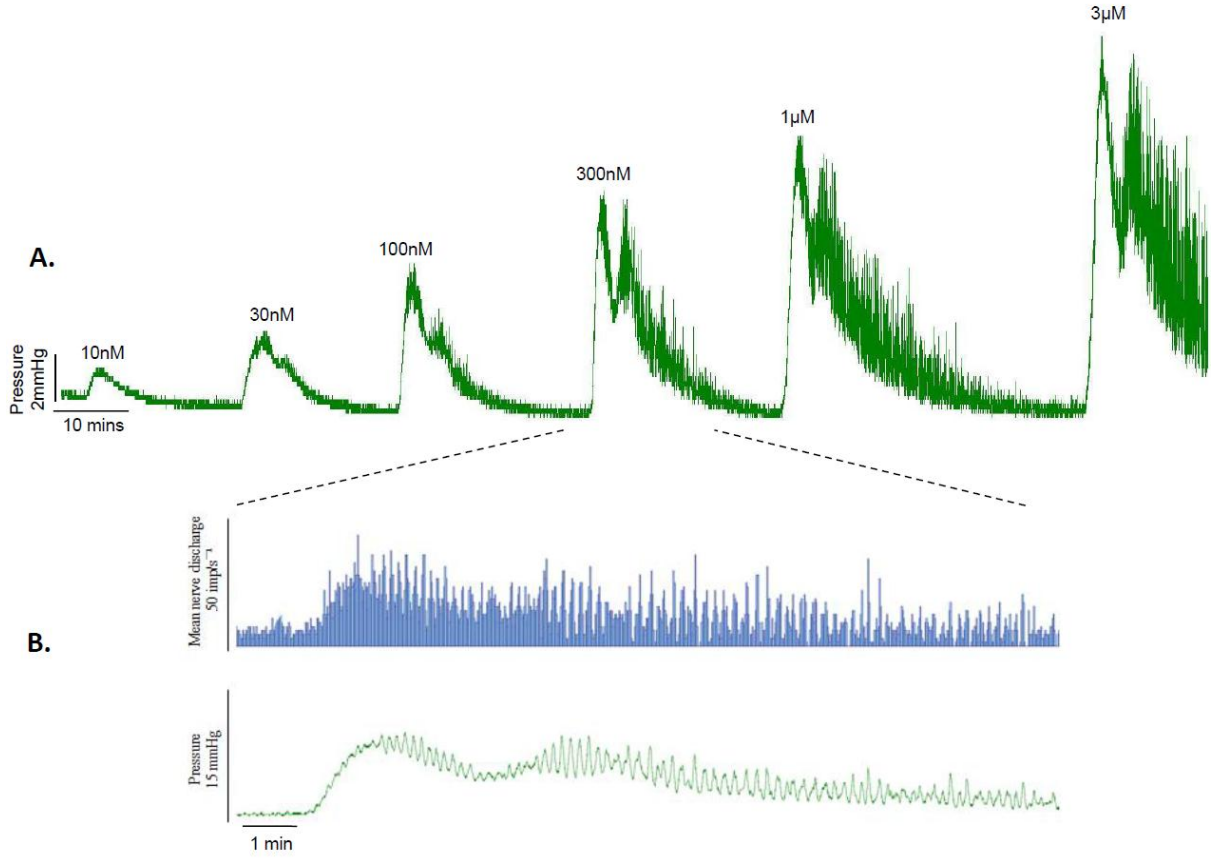

Fig 5.3.1 A, *Experimental trace of intravesical pressure in response to increasing concentrations of NKA (10nM-3μM) applied to the serosal surface of a whole bladder.* ***B,*** *Intravesical pressure and afferent nerve responses to NKA (300nM). The phasic bladder contractions and afferent nerve activity are temporally linked.*

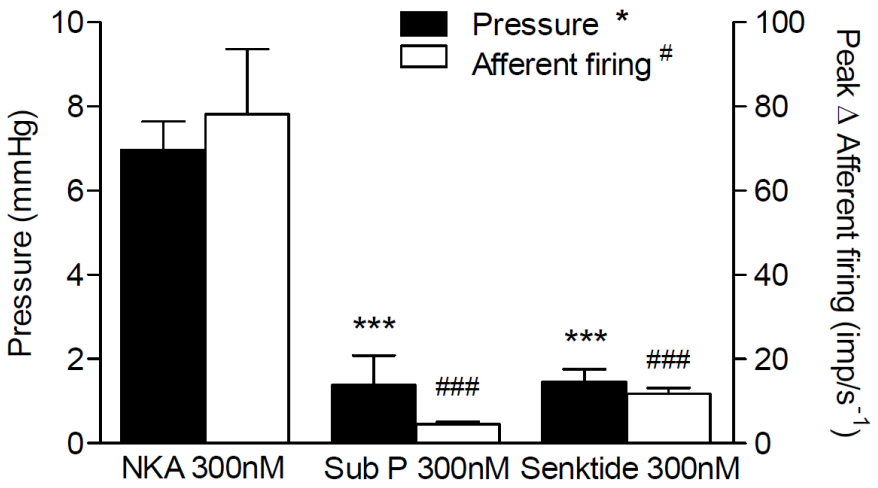

Fig 5.3.2 *Mean±(SEM) peak afferent firing and intravesical pressure increase in response to tachykinin receptor agonists NKA (300nM, n=6), substance P (300nM, n=4), senktide (300nM, n=4), (***p≤0.001, ###p≤0.001 compared to NKA).*

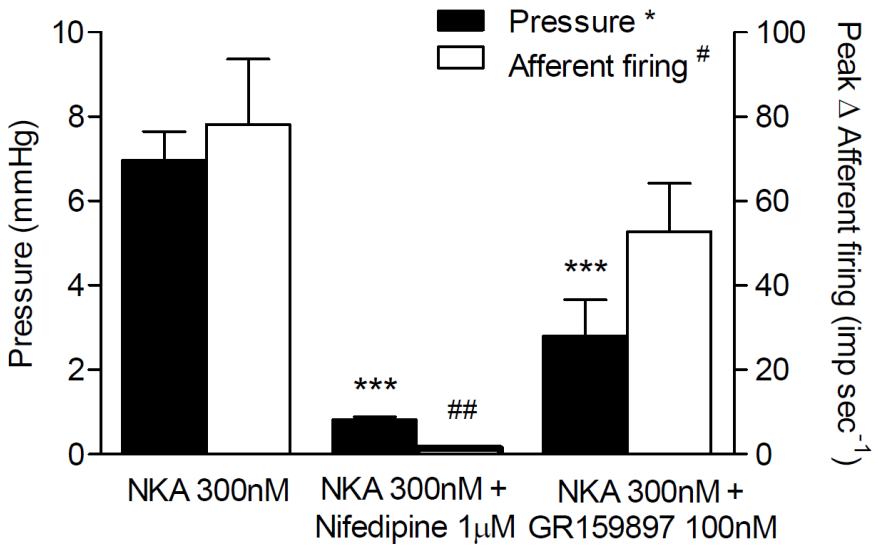

Fig 5.3.3 *Mean±(SEM) peak afferent firing and intravesical pressure increase in response to NKA (300nM, n=6) in the presence and absence of nifedipine (1μM, n=6) and NK2 receptor antagonist GR159897 (100nM, n=4), (***p≤0.001, ##p≤0.01, compared to NKA).*

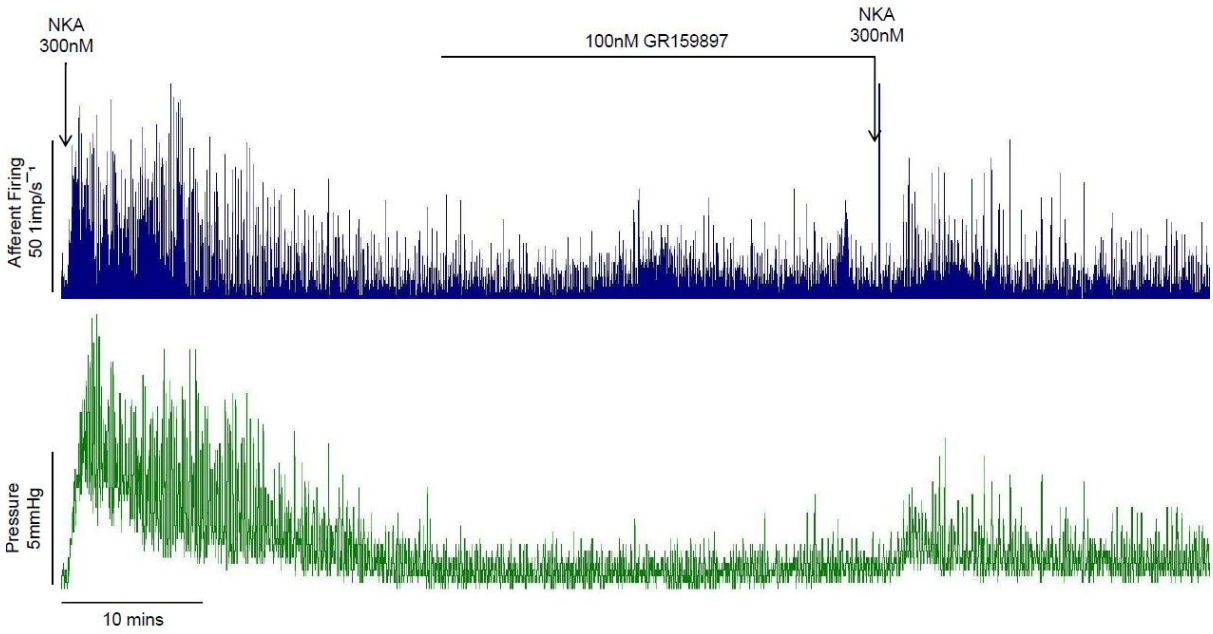

Fig 5.3.4 *Experimental trace of NKA (300nM) induced afferent nerve activity (imp/s^{-1}) and intravesical pressure (mmHg) prior to, and in the presence of the NK2 antagonist GR159897 (100nM).*

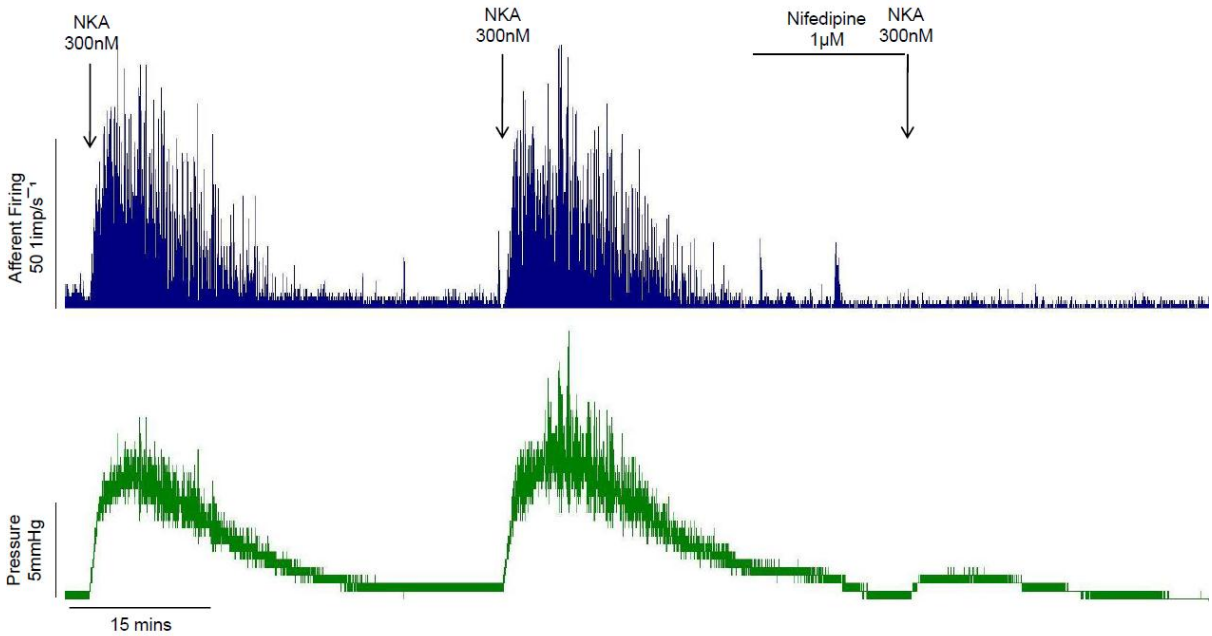

Fig 5.3.5 *Experimental trace of NKA (300nM) induced afferent nerve activity (imp/s^{-1}) and intravesical pressure (mmHg) prior to, and in the presence of the nifedipine (1μM).*

5.4 THE EFFECTS OF EXTRALUMINAL TACHYKININ RECEPTOR AGONISTS ON AFFERENT NERVE AND DETRUSOR CONTRACTION DURING BLADDER DISTENSION

As the bladder fills with saline there is a rise in intraluminal pressure and mechanosensitive afferent fibers in the bladder wall activate afferent nerves. The rise in pressure reflects the ability of the detrusor muscle to stretch in order to accommodate the increase in volume and gives rise to a pressure/volume curve that is an indication of bladder compliance. By examining the effect of tachykinin agonists on the pressure/volume relationship and corresponding changes in the stimulus/response function of the bladder afferents it is possible to determine the relative effect of the various tachykinin receptors on afferent and detrusor activity during bladder distension. All compliance curves and nerve/pressure relationships are analysed by two-way ANOVA with Bonferroni multiple comparisons post-hoc test.

With the bladder empty and draining freely extraluminal application of the NK1 receptor agonist substance P (300nM) had no immediate effect on afferent discharge. In addition, in the continued presence of substance P the response to ramp distension was unchanged. Therefore, there was no significant effect on the pressure/volume response profile ($p \geq 0.05$, n=6, **fig 5.4.2)**. Moreover, the increase in afferent nerve activity that accompanied the increase in intravesical pressure was also not significantly different during bladder distension ($p \geq 0.05$, n=6, **fig 5.4.3**). These results show that substance P has no significant effect on either muscle compliance or the pressure/nerve response to bladder distension. From these results it can be concluded that substance P (and therefore the NK1 receptor) has no influence on mouse bladder distension responses.

The same parameters were examined with extraluminal incubation with the NK3 receptor agonist senktide (300nM). Senktide (300nM) had no significant effect on muscle compliance ($p \geq 0.05$, n=6, **fig 5.4.6**), nor afferent nerve discharge in response to bladder distension ($p \geq 0.05$, n=6, **fig 5.4.7**). From these results it can be concluded that senktide (and therefore the NK3 receptor) has no influence on mouse bladder distension responses.

In contrast to the NK1 and NK3 receptor agonists, extraluminal incubation with the NK2 receptor agonist NKA (300nM) mediates a significant decrease in the compliance of the muscle over the time course of bladder distension ($p \leq 0.01$, n=6, **fig 5.4.9**) which was more apparent at low pressures. Despite an NKA induced change in detrusor compliance, bath applied NKA (300nM) had no significant effect on pressure/nerve relationship during bladder distension ($p \geq 0.05$, n=6, **fig 5.4.11**).

The change in detrusor muscle compliance can be seen in the experimental trace of **fig 5.4.8**. Further investigation of the role of the NK2 receptor in bladder compliance showed that bath applied incubation with the NK2 receptor antagonist GR159897 (100nM) significantly increases bladder compliance over the time course of bladder distension ($p \leq 0.001$, n=5, **Fig 5.4.10**), with the effects again occurring at lower bladder pressures. Bath applied GR159897 (100nM) was also able to antagonise the actions of NKA (30nM) on compliance confirming an NK2 receptor interaction.

Extravesical Substance P

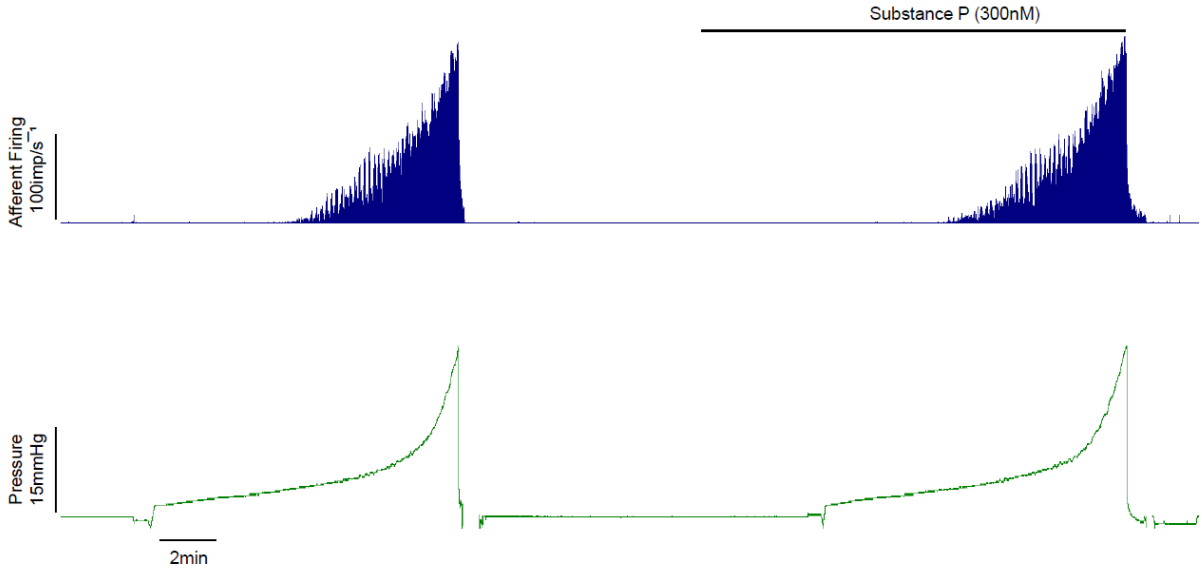

Fig 5.4.1 *Experimental trace showing intravesical pressure (mmHg) and afferent nerve (imp/s⁻¹) responses to bladder distension prior to, and during substance P (300nM) incubation.*

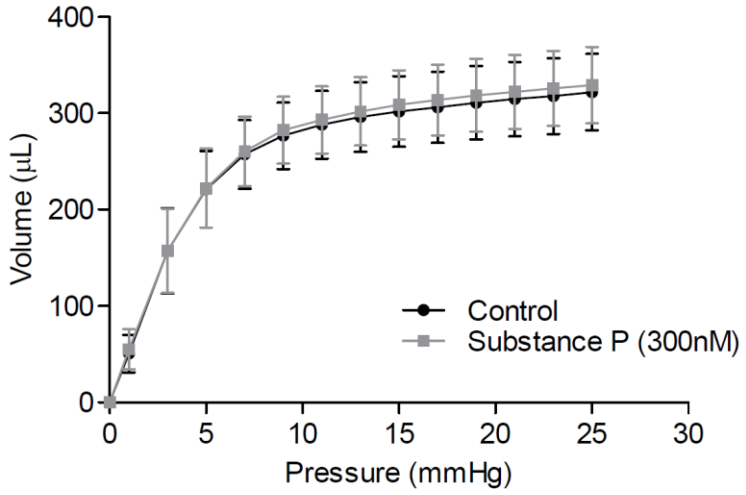

Fig 5.4.2 *Mean±(SEM) pressure/volume relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extaluminal substance P (300nM), (n=6).*

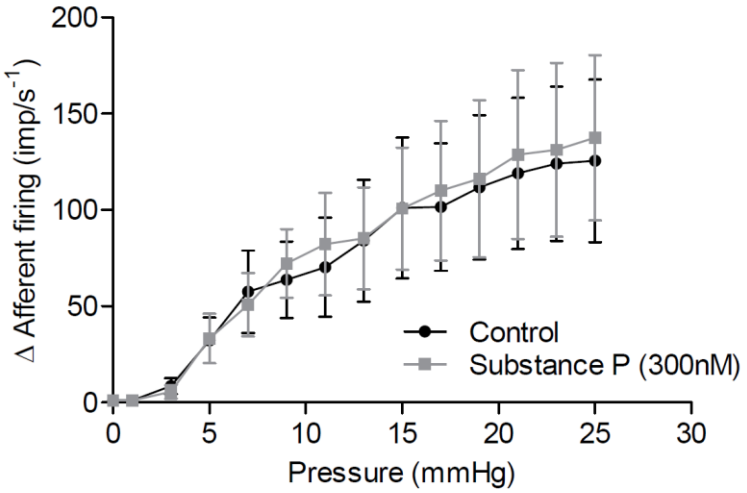

Fig 5.4.3 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal substance P (300nM), (n=6).*

Extravesical Senktide

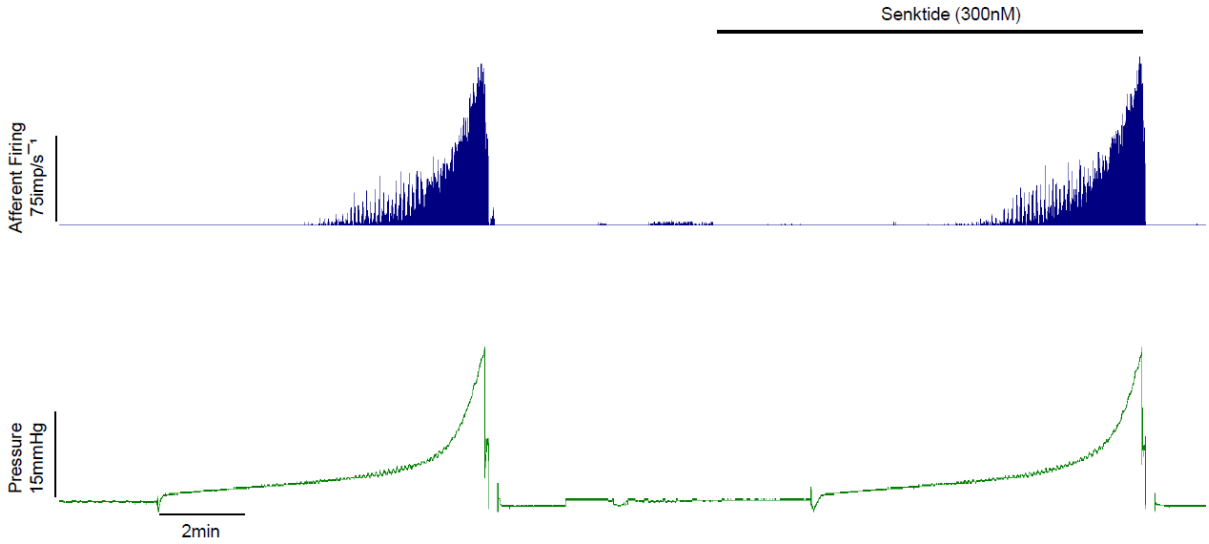

Fig 5.4.5 *Experimental trace showing intravesical pressure (mmHg) and afferent nerve (imp/s⁻¹) responses to bladder distension prior to, and during senktide (300nM) incubation.*

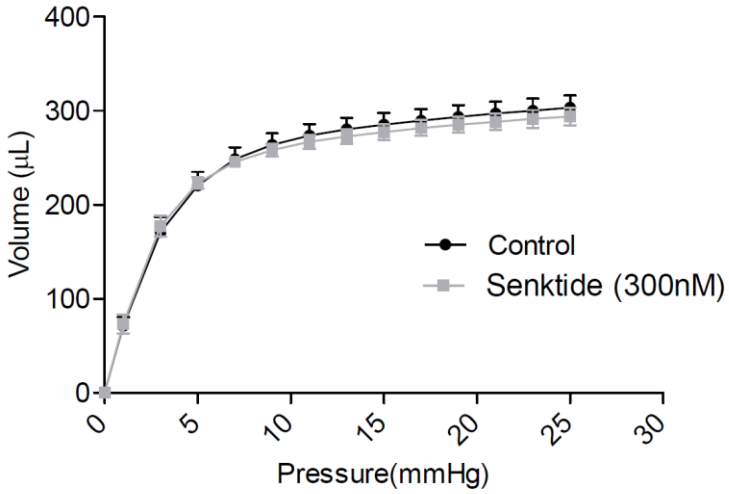

Fig 5.4.6 *Mean±(SEM) pressure/volume relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal senktide (300nM), (n=6).*

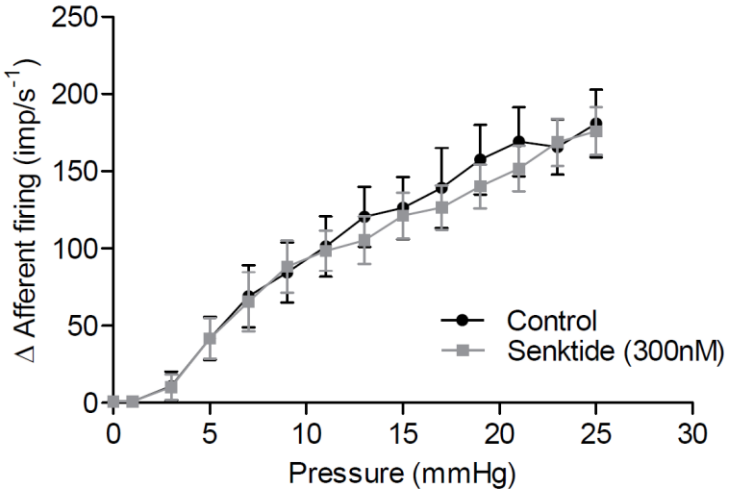

Fig 5.4.7 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal senktide (300nM), (n=6).*

Extravesical NKA

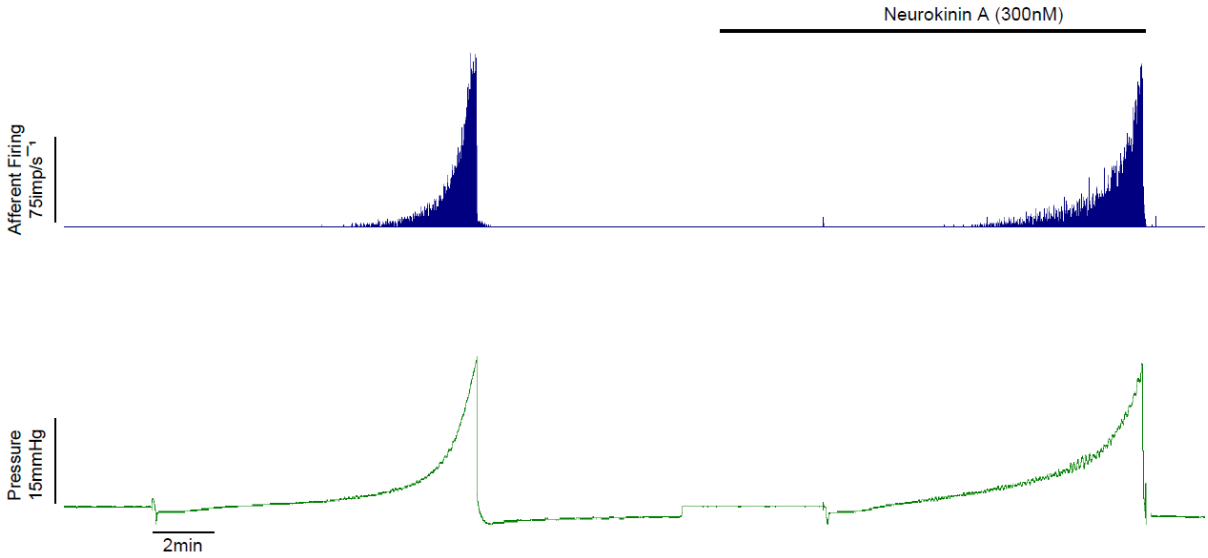

Fig 5.4.8 *Experimental trace showing intravesical pressure (mmHg) and afferent nerve (imp/s⁻¹) responses to bladder distension prior to, and during NKA (300nM) incubation. There is no observed contraction due to the initial addition of NKA (300nM) to the bath, as the outflow tap is open and there is no pressure inside the bladder.*

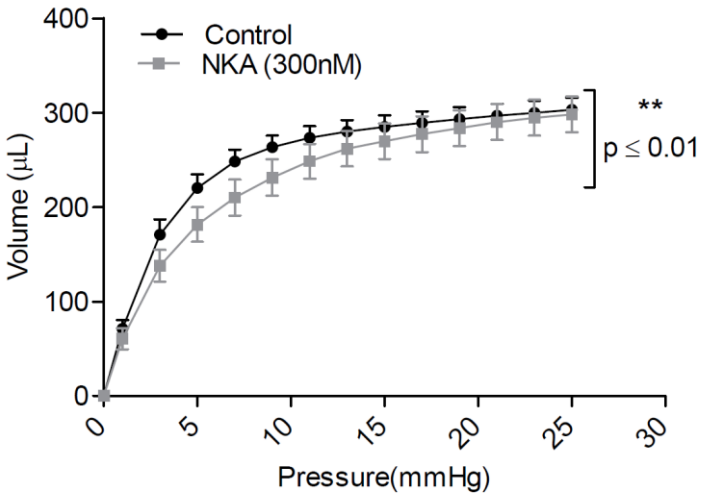

Fig 5.4.9 *Mean±(SEM) pressure/volume relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal NKA (300nM), (**p≤0.01, n=6).*

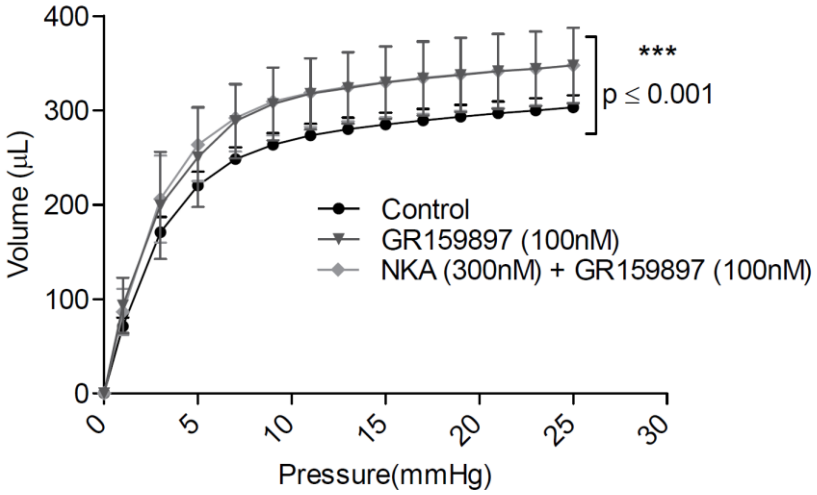

Fig 5.4.10 *Mean±(SEM) pressure/volume relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal GR159897 (100nM, n=5) or GR159897 (100nM) + NKA (300nM, n=5), (***p≤0.001 GR159897 Vs control).*

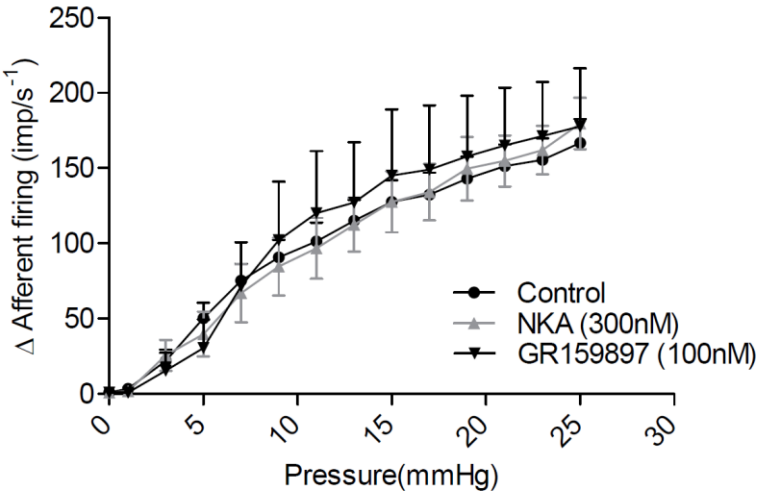

Fig 5.4.11 *Mean(±SEM) pressure/nerve relationship within the bladder when distended with saline at a rate of 30µl/min in the presence of extraluminal GR159897 (100nM, n=5) or NKA (300nM, n=6).*

5.5 NKA INDUCED EFFECTS ON PURINERGIC ACTIVATION

ATP is released from the urothelium during stretch and is hypothesised to mediate bladder mechanotransduction via purinoceptors on afferent nerves. To investigate the ability of the tachykinin NK2 receptor to modulate purinergic mediated afferent nerve activity in the mouse bladder, the effect of extraluminally incubated NKA was investigated on purinergic evoked detrusor contraction and afferent nerve activity. A concentration of NKA was chosen (1nM) for which no contraction of the smooth muscle occurred.

Bath application of the purinergic agonist αβMe-ATP (30μM) causes a significant increase in afferent nerve activity and concurrent detrusor smooth muscle contraction (**fig 5.5.1**) which is characterised by an initial sharp increase in afferent nerve activity which returns to baseline over 2-3 minutes. Application of a sub-contraction threshold dose of NKA (1nM) has no effect on αβMe-ATP (30μM) induced afferent firing ($p \geq 0.05$, n=6, **Fig 5.5.2**) but significantly increases the magnitude of detrusor smooth muscle contraction throughout the time course of the response ($p \leq 0.01$, n=6, two-way ANOVA Bonferroni multiple comparisons post-hoc test, **fig 5.5.3**). A significant potentiation of the maximal contraction response to αβMe-ATP is seen with NKA (1nM) (14.5±1.9 Vs 18.0±1.5mmHg, $p \leq 0.05$, n=6) which is blocked by the NK2 antagonist GR159897 (100nM) (14.5±1.9 Vs 16.2±0.9mmHg, $p \geq 0.05$, n=6, one-way ANOVA, Bonferroni multiple comparisons post-hoc test, **fig 5.5.4**).

These results indicate that although a sub-contraction concentration of NKA is unable to modulate afferent nerve responses to αβMe-ATP, and thus is unlikely to influence the indirect component of mechanotransduction via urothelial ATP release, there is a mechanism whereby activation of the NK2 receptor is able to mediate the purinergic component of detrusor contraction which becomes increasingly important during lower urinary tract disorders.

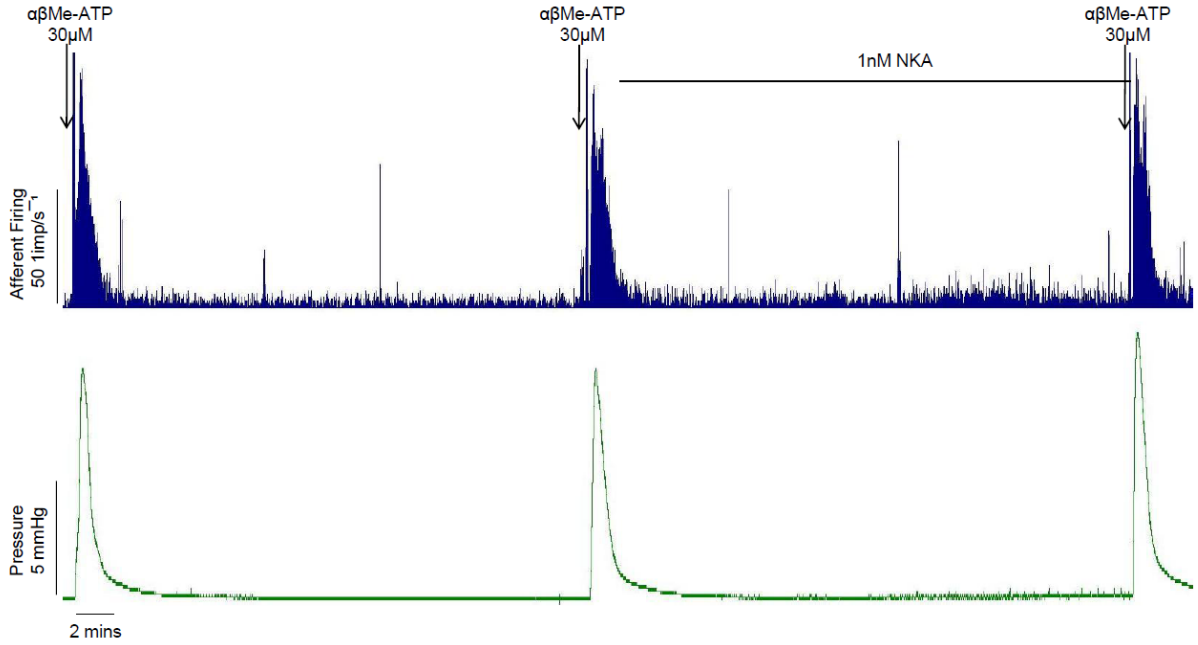

Fig 5.5.1 *Example trace of αβMe-ATP (30μM) induced afferent nerve activity and detrusor smooth muscle contraction in the presence and absence of a sub-contraction concentration of NKA (1nM). These experiments were performed in a bladder predistended to 12mmHg in order to accurately measure contraction responses.*

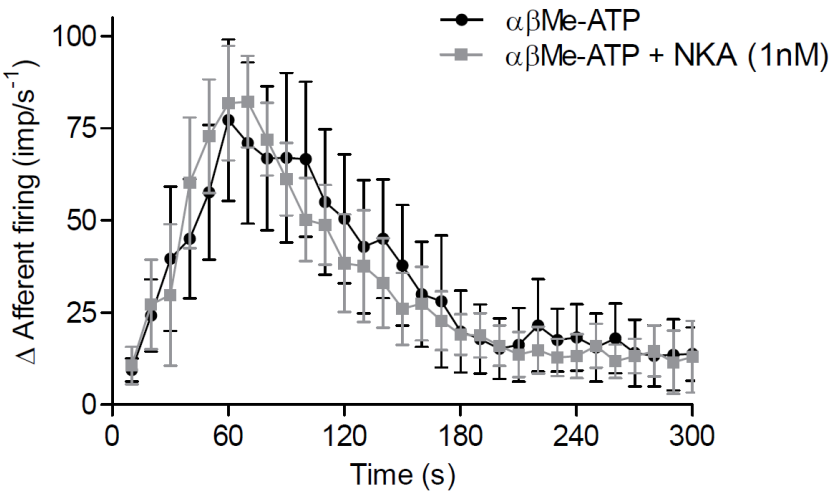

Fig 5.5.2 *Mean±(SEM) afferent nerve activity in response to αβMe-ATP (30μM) in the absence and presence on NKA (1nM), (n=6).*

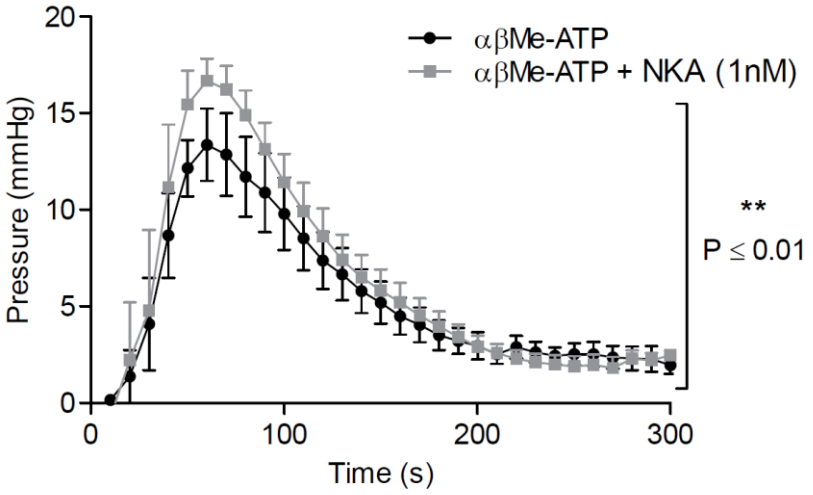

Fig 5.5.3 *Mean±(SEM) intravesical pressure in response to αβMe- ATP (30μM) in the absence and presence on NKA (1nM), (**p≤0.001, n=6).*

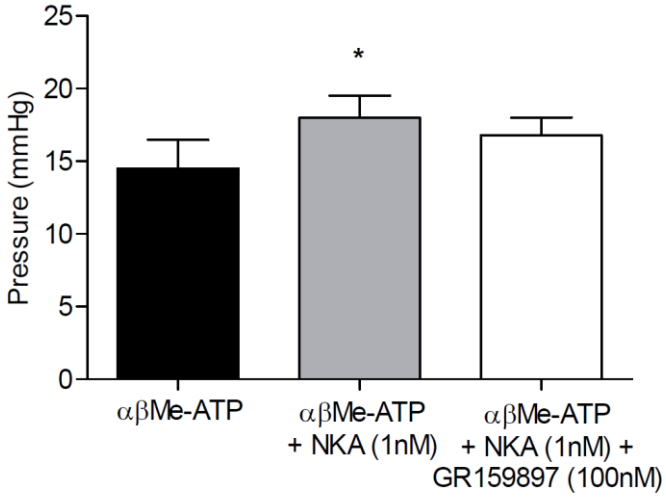

Fig 5.5.4 *Mean±(SEM) peak intravesical pressure in response to αβMe-ATP (30μM, n=6) in the presence of NKA (1nM), (p≤0.05 Vs control, n=6), and NKA (1nM) + GR159897 (100nM).*

5.6 THE EFFECTS OF INTRAVESICAL TACHYKININ RECEPTOR AGONISTS ON DETRUSOR SMOOTH MUSCLE AND AFFERENT NERVE ACTIVITY

The previous sub-sections concentrated on the extra luminal application of tachykinin agonists. Under these circumstances the agonists will have access to cellular targets throughout the bladder wall. As such, direct effects on the muscle wall may overwhelm any modulating influence following actions at the level of the urothelium. Therefore, in order to investigate the ability of the urothelium to respond to tachykinin receptor agonists and their ability to influence detrusor smooth muscle compliance and afferent nerve activity, tachykinin receptor agonists were perfused intraluminally into the bladder in the following experiments. All compliance curves and nerve/pressure relationships are analysed by two-way ANOVA with Bonferroni multiple comparisons post-hoc test.

Similar to extraluminal application, intravesical instillation of substance P (300nM) had no significant effect on detrusor smooth muscle compliance ($p \geq 0.05$, n=5, **fig 5.6.2**) throughout the time course of bladder distension. There was also no significant effect of substance P (300nM) on the pressure/nerve response during the time course of bladder distension ($p \geq 0.05$, n=5, **fig 5.6.3**).

Likewise, intravesical instillation of senktide (300nM) had no significant effect on the compliance of detrusor smooth muscle ($p \geq 0.05$, n=5, **fig 5.6.5**) throughout the time course of bladder distension. Intravesical perfusion of senktide (300nM) also had no significant effect on the distension evoked pressure/nerve relationship ($p \geq 0.05$, n=5, **fig 5.6.6**).

In contrast, intravesical perfusion of NKA (300nM) significantly decreased the compliance of the detrusor smooth muscle to an increases in intravesical volume throughout the time course of bladder distension when compared to control ($p \leq 0.0001$, n=6, **fig 5.6.7**). Intravesical perfusion of the NK2 receptor antagonist GR159897 (100nM) alone had no significant effect on bladder compliance during bladder distension ($p \geq 0.05$, n=6), however, GR159897 (100nM) was able to reverse NKA (300nM) induced decreases in bladder compliance ($p \geq 0.05$, n=6, **fig 5.6.8**).

Analysis of afferent nerve firing showed intravesical perfusion of NKA (300nM) has no significant effect on the pressure/nerve relationship during bladder distension ($p \geq 0.05$, n=6, **fig 5.6.9**). Intravesical perfusion of either GR159897 (100nM) alone, or GR159897 + NKA (300nM) were also without effect on the pressure nerve response during bladder distension ($p \geq 0.05$, n=6, **5.6.10**). The analysis of the pressure/nerve relationship during distension provides essential information

regarding the overall state of the bladder, however as **fig 5.7.1** shows, this type of analyses masks subtle differences when considering afferent nerve firing in response to intravesical NKA and the next subsection will delve deeper into the analysis concerned with this.

Intravesical Sub P

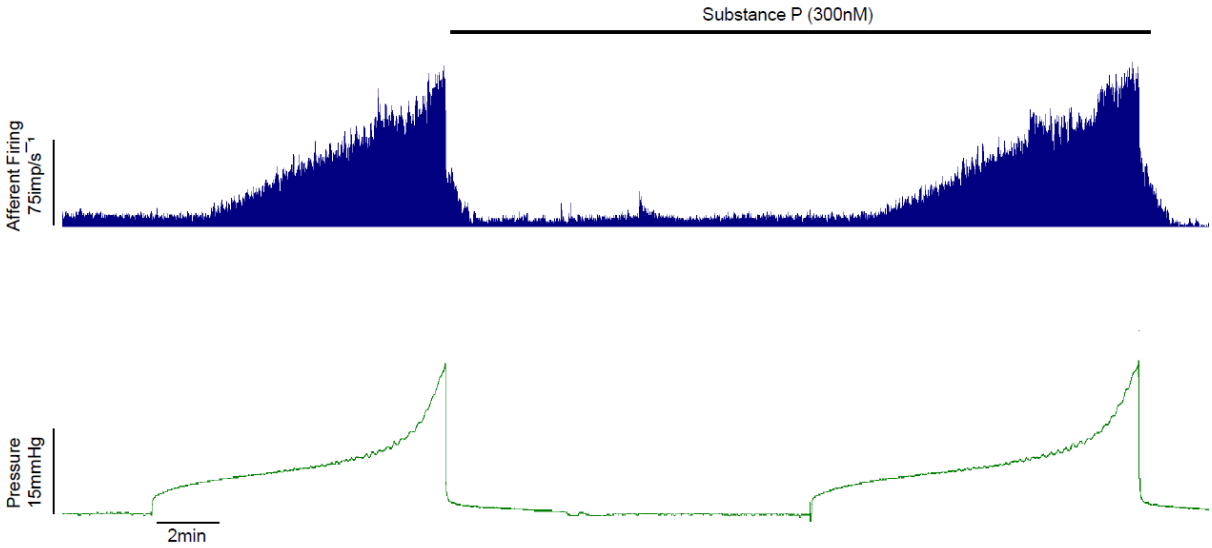

Fig 5.6.1 *Experimental trace showing intravesical pressure (mmHg) and afferent nerve (imp/s^{-1}) responses to bladder distension prior to, and during intraluminal perfusion with substance P (300nM).*

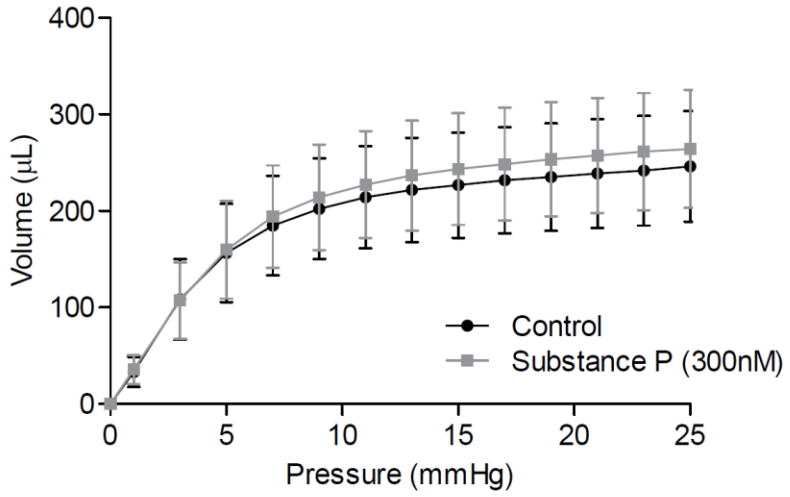

Fig 5.6.2 *Mean±(SEM) pressure/volume relationship of the bladder when distended with either saline or substance P (300nM) at a rate of 30µl/min, (n=5).*

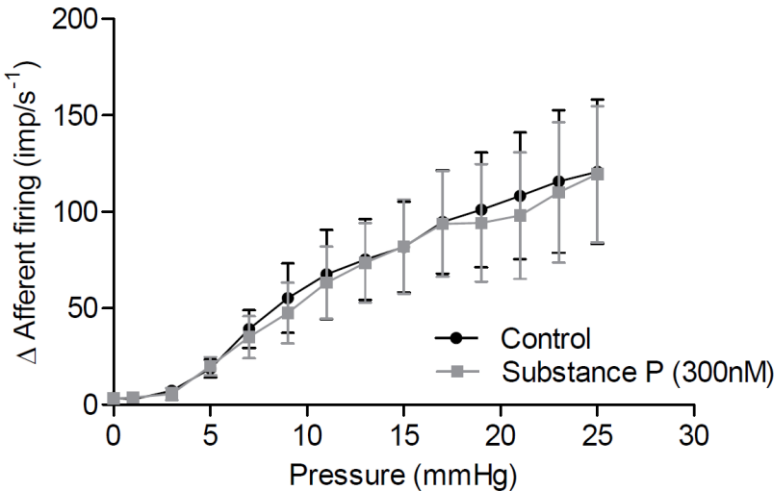

Fig 5.6.3 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with either saline or substance P (300nM) at a rate of 30µl/min, (n=5).*

Intravesical Senktide

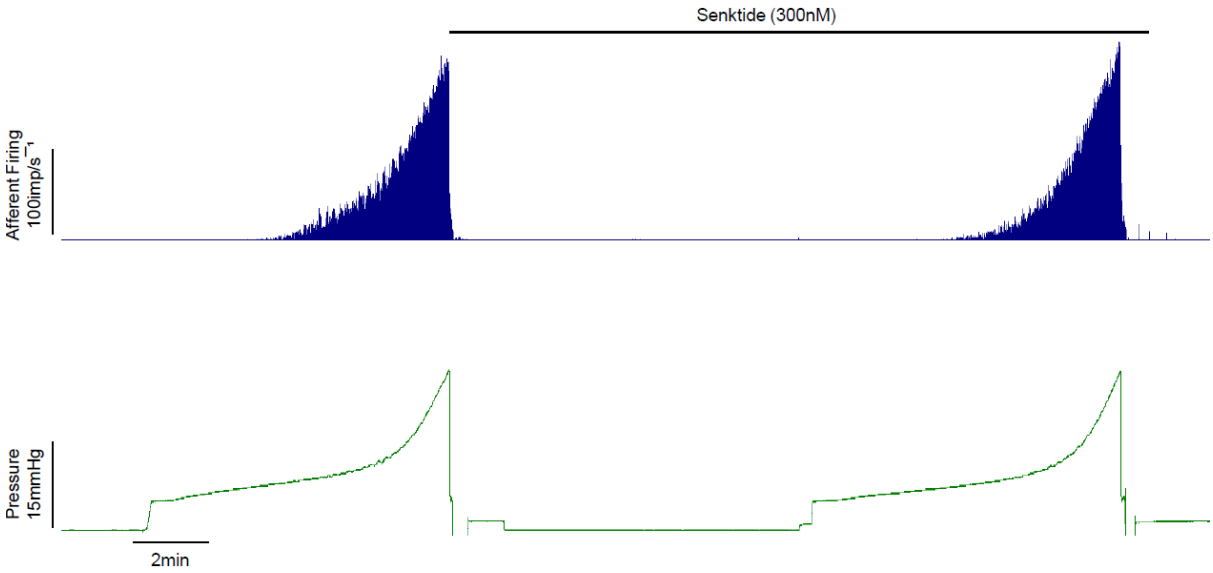

Fig 5.6.4 *Experimental trace showing intravesical pressure (mmHg) and afferent nerve (imp/s⁻¹) responses to bladder distension prior to, and during intraluminal perfusion with senktide (300nM).*

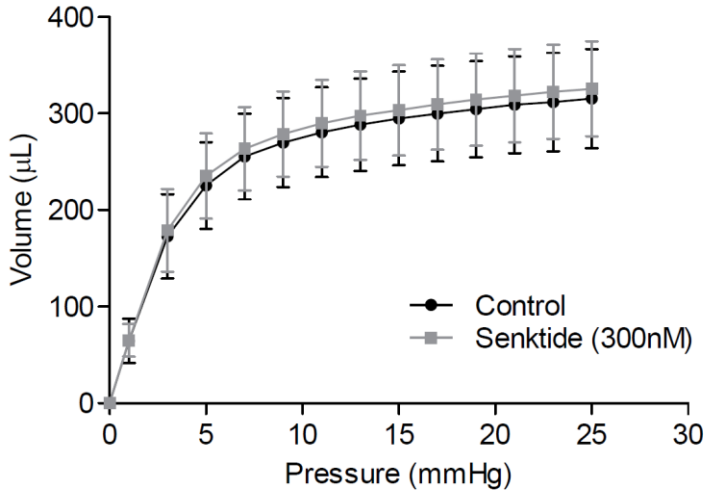

Fig 5.6.5 *Mean±(SEM) pressure/volume relationship of the bladder when distended with either saline or senktide (300nM) at a rate of 30µl/min, (n=5).*

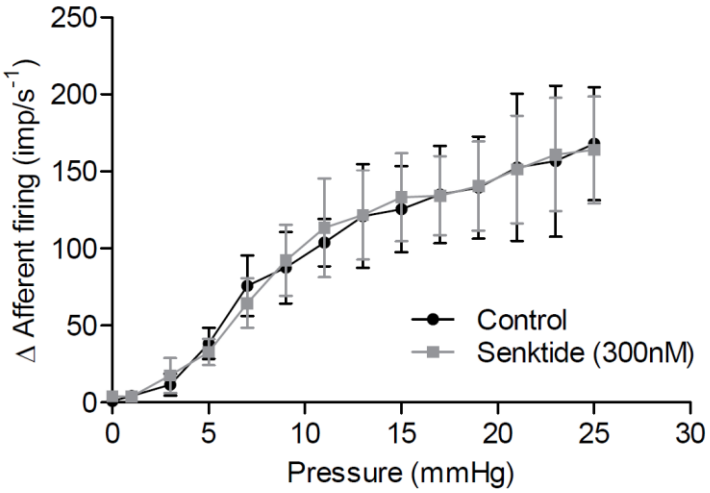

Fig 5.6.6 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with either saline or senktide (300nM) at a rate of 30µl/min, (n=5).*

Intravesical NKA

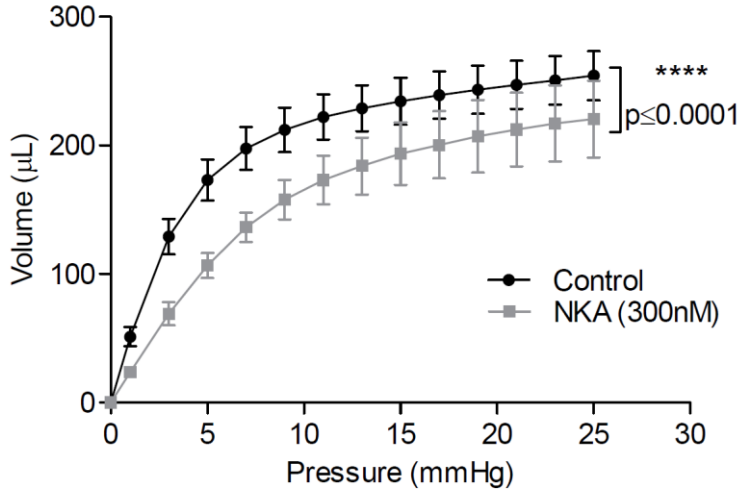

Fig 5.6.7 *Mean±(SEM) pressure/volume relationship of the bladder when distended with either saline or NKA (300nM, n=6) at a rate of 30µl/min, (****p≤0.0001 Vs Control)*

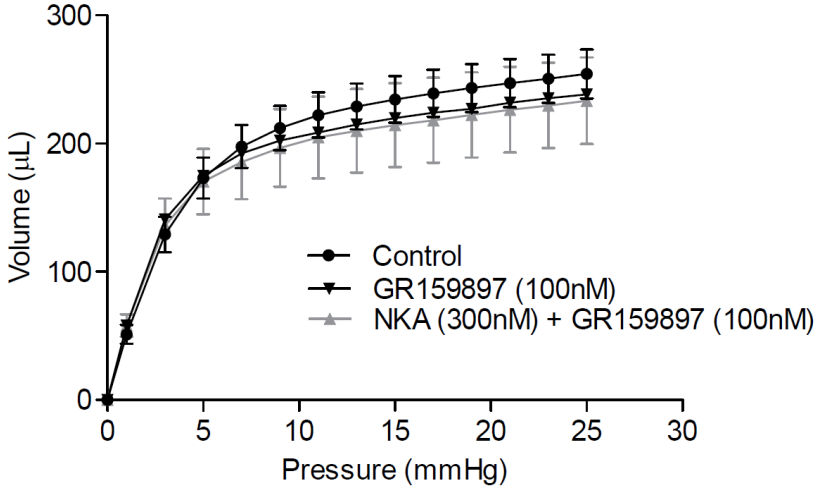

Fig 5.6.8 *Mean±(SEM) pressure/volume relationship of the bladder when distended with either saline or the NK2 antagonist GR159897 (100nM, n=6) or NKA (300nM + GR159897 100nM, n=6) at a rate of 30µl/min.*

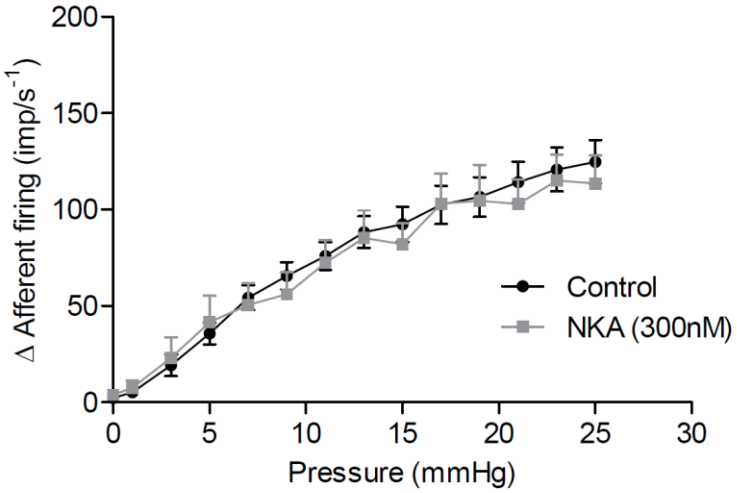

Fig 5.6.9 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with either saline or NKA (300nM, n=6) at a rate of 30µl/min.*

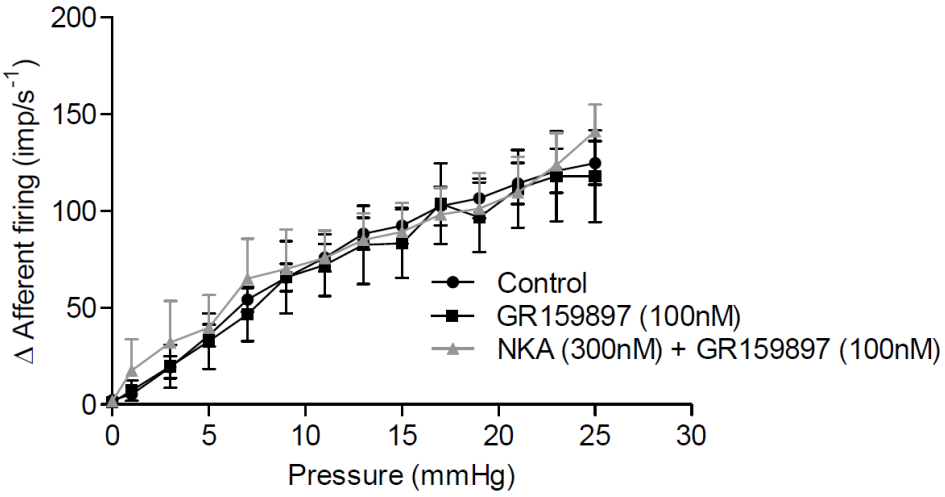

Fig 5.6.10 *Mean±(SEM) pressure/nerve relationship within the bladder when distended with either saline, GR159897 (100nM, n=6), or GR159897 + NKA (300nM, n=6) at a rate of 30µl/min.*

5.7 NKA INDUCED SPONTANEOUS DETRUSOR ACTIVITY

There is a degree of phasic detrusor muscle contraction during normal bladder distension which is of low frequency and amplitude. Accompanying these spontaneous detrusor contractions are bursts of afferent nerve activity. The pattern of contractile activity and associated afferent discharge were significantly altered with the intraluminal perfusion of NKA (300nM, **fig 5.7.1**). Since these changes occur at physiological levels of distension it is feasible that this could impact on sensory mechanotransduction within the bladder.

Intravesical instillation of substance P (300nM) and senktide (300nM) had no significant effect on the amplitude or frequency of distension induced spontaneous detrusor contractions compared to control ($p \geq 0.05$, n=5, **fig 5.7.2, 5.7.3**). In contrast intravesical instillation of NKA (300nM, n=6) caused a significant increase in the amplitude (0.9±0.1 Vs 2.2±0.4mmHg, $p \leq 0.001$, n=6) and frequency (6.2±1 Vs 22.2±3.7/distension, $p \leq 0.001$, n=6, one-way ANOVA, Dunnett's multiple comparisons post-hoc test) of spontaneous contractions, which were abolished with prior instillation of GR159897 (100nM). An experimental trace showing detrusor contractions and bursts of afferent nerve activity can be seen in **fig 5.7.4** and **fig 5.7.5.**

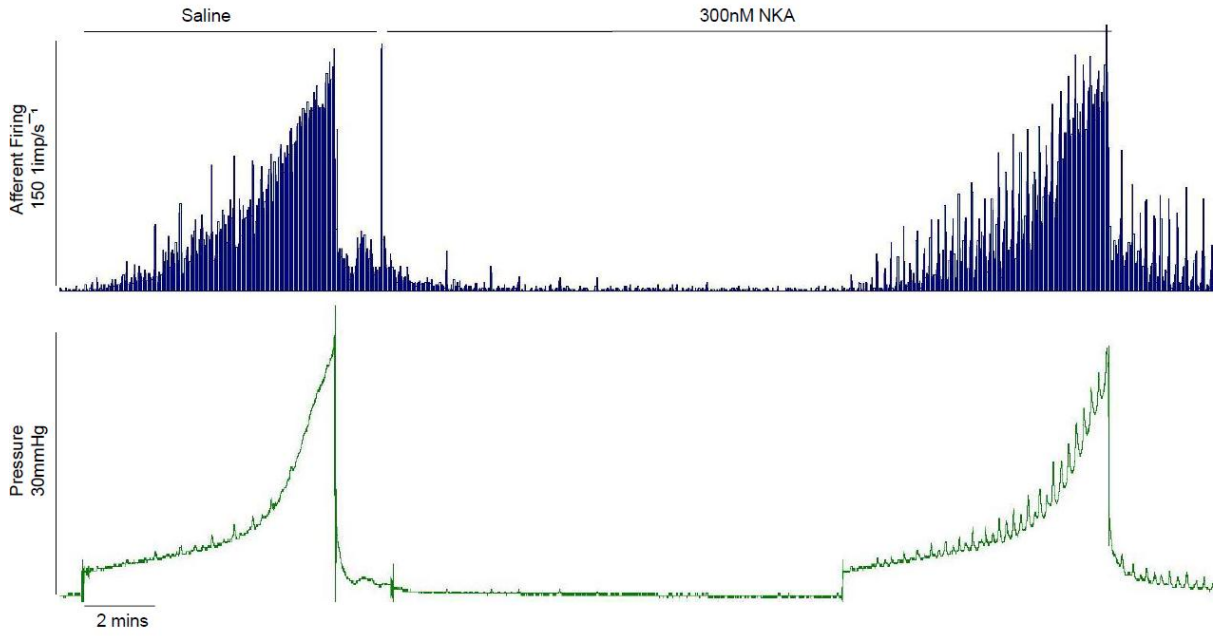

Fig 5.7.1 *Example trace of intraluminal NKA (300nM) induced phasic detrusor contractions with accompanying afferent nerve activity during bladder distension.*

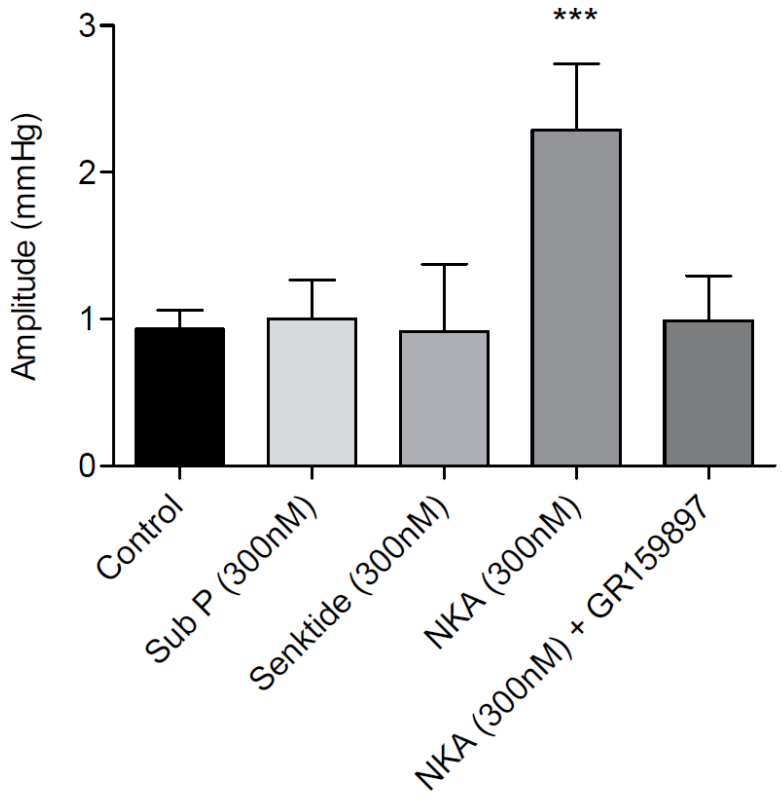

Fig 5.7.2 *Mean±(SEM) amplitude of contractions (mmHg) evoked by bladder distension with intravesical substance P (300nM, n=5), senktide (300nM, n=5), NKA (300nM, n=6) and the interactions of GR159897 (100nM, n=6), (***p≤0.001 Vs control).*

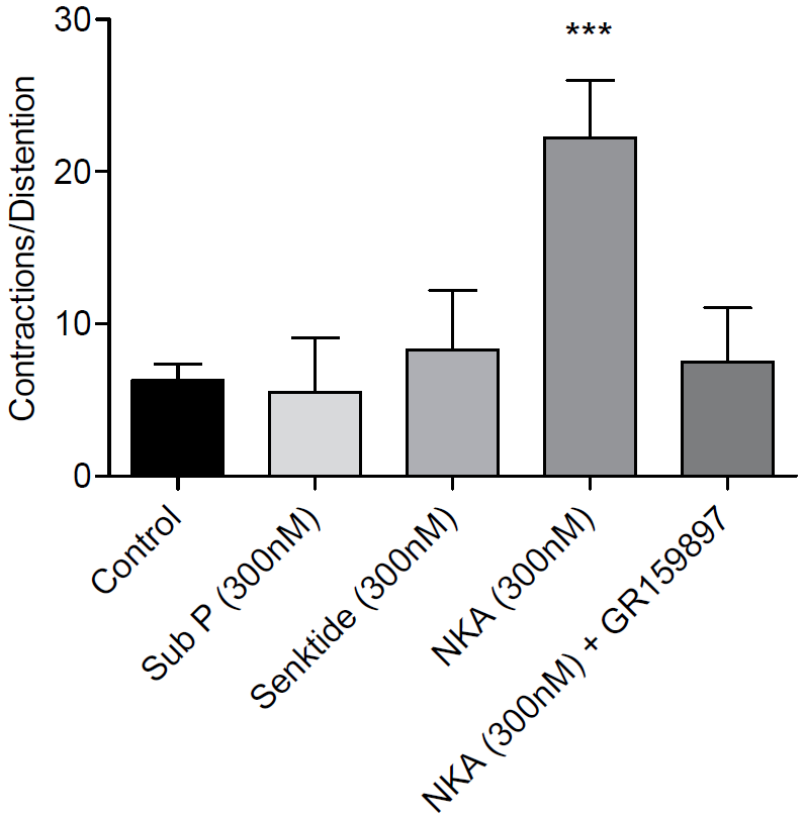

Fig 5.7.3 *Mean±(SEM) frequency (contractions/distension) of spontaneous contractions elicited by bladder distension with intravesical substance P (300nM, n=5), senktide (300nM, n=5), NKA (300nM, n=6) and the interactions of GR159897 (100nM, n=6), (***p≤0.001 Vs control).*

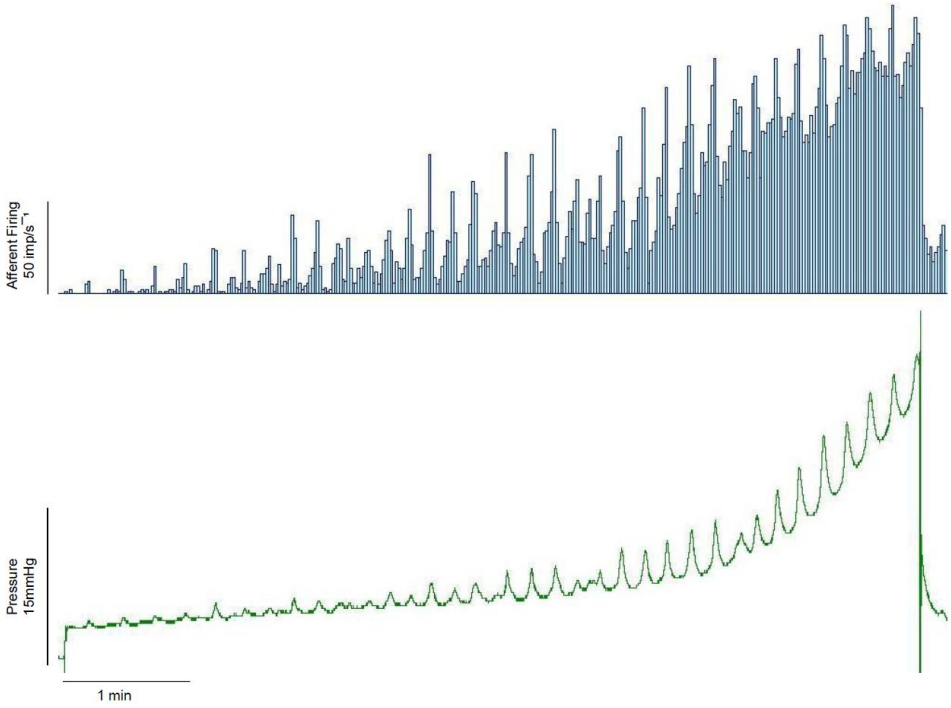

Fig 5.7.4 *Experimental trace showing spontaneous detrusor contractions and accompanying afferent nerve activity during bladder distension with intraluminal NKA (300nM).*

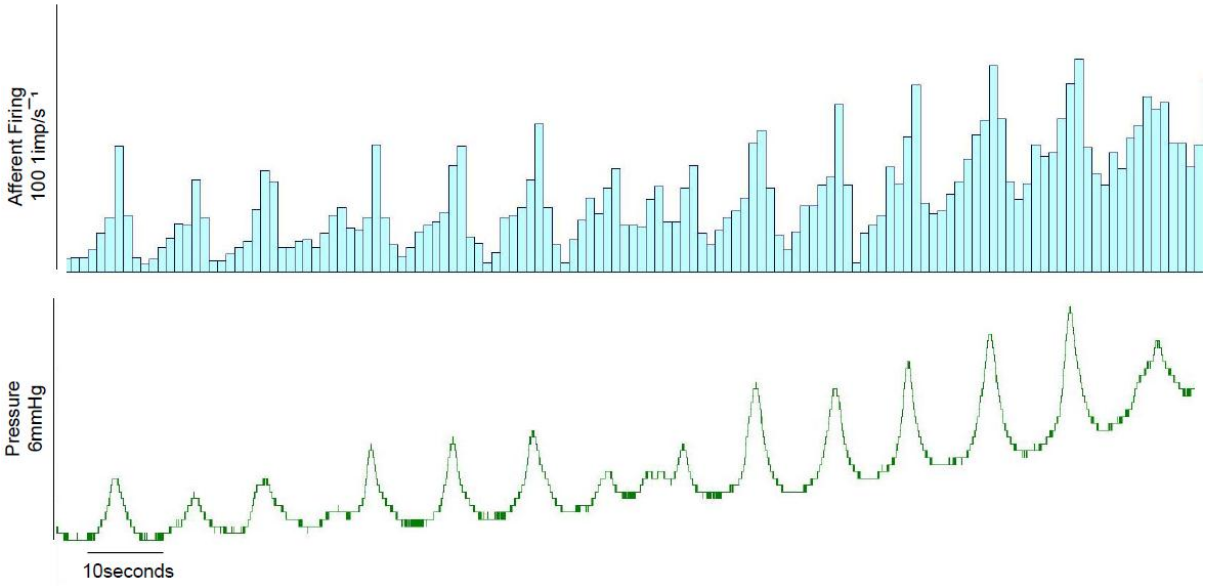

Fig 5.7.5 *Spontaneous detrusor contractions and associated afferent nerve activity during bladder distension with intraluminal NKA (300nM). Zoom of* ***fig 6.7.4****.*

5.8 SPONTANEOUS AFFERENT ACTIVITY OF THE BLADDER IN RESPONSE TO INTRALUMINAL TACHYKININS

In order to investigate the relationship between intraluminal instillation of tachykinins and afferent firing with no effect from detrusor contraction, tachykinins were instilled in the bladder lumen and the spontaneous afferent discharge was observed.

Intravesical perfusion of tachykinin agonists substance P (300nM), senktide (300nM), and NKA (300nM) had no significant effect on spontaneous afferent activity although there was a trend towards an increase with NKA which did not reach statistical significance (**fig 5.8.2**). The experimental trace in **fig 5.8.1** shows examples of the relationship between the raw nerve activity and the rate histogram of afferent nerve activity imp/s^{-1}. No observable differences can be seen.

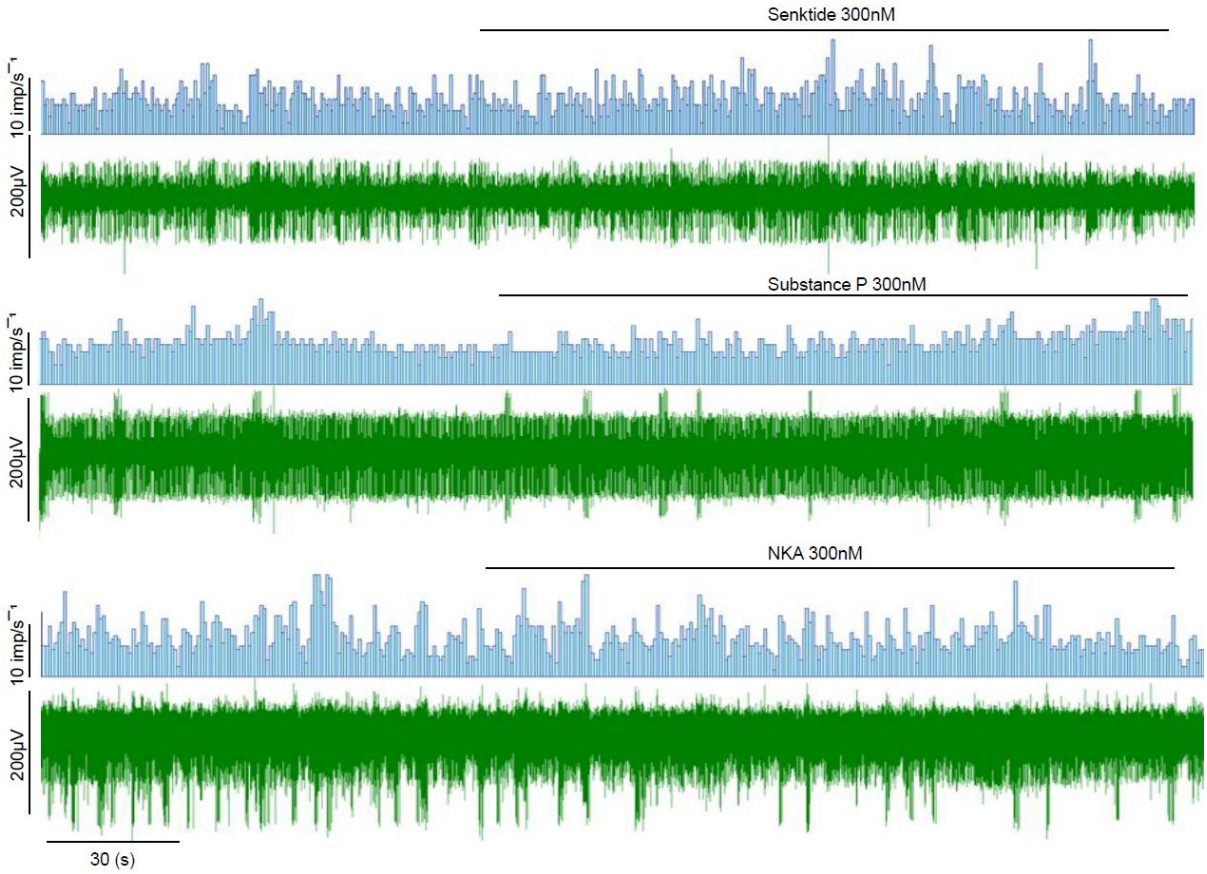

Fig 5.8.1 Experimental traces representing the effects of intravesical instillation of tachykinin agonists senktide (300nM), substance P (300nM) and NKA (300nM) on baseline spontaneous afferent activity. For each, the upper trace is a rate histogram of nerve activity (imp/s^{-1}) and below is the raw nerve activity.

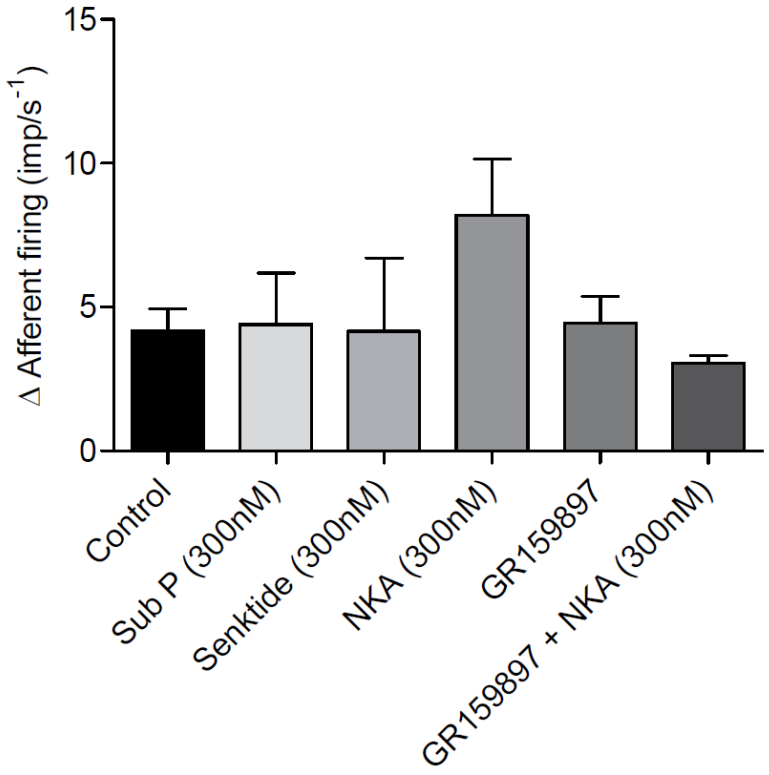

Fig 5.8.2 *Mean±(SEM) average baseline spontaneous afferent activity recorded over 100s before, and following intravesical instillation of tachykinin agonists substance p (300nM, n=5), senktide (300nM, n=5), and NKA (300nM, n=6).*

5.9 NKA INDUCED EFFECTS ON THE UROTHELIUM

The results above indicate that intravesical NKA is able to induce changes in bladder mechanosensitivity secondary to changes in detrusor smooth muscle function. It was also found that there was no significant effect of intravesical tachykinins on afferent nerve discharge. Thus, there is potential for the effects of intravesical NKA to be mediated by a direct effect on the urothelium. For this to occur, tachykinin receptors must be present within the urothelial cell layers.

Quantification of tachykinin receptor mRNA was performed on primary mouse urothelial cells (PMUCs) and results are expressed relative to β-actin. Urothelial cells expressed all three of the known tachykinin receptors (**fig 5.9.1**). There was relatively equal expression of both NK1 and NK2 receptor mRNA, and an approximately two-fold greater expression of NK3 receptor mRNA.

Identification of the mRNA coding for the tachykinin receptors, together with prior experiments showing the ability of intravesically instilled NKA to significantly alter normal bladder function led to the proposal that urothelial cells were able to respond directly to NKA. This was investigated by examining the ability of NKA to alter mediator release from PMUCs.

ATP, ACh, and prostaglandin E_2 were released into the lumen of the bladder at rest, and in the case of ATP, significantly higher amounts were released following bladder distension (**fig 5.9.3**) (2.3±0.3 Vs 6.2±1.5nM, p≤0.01, n=6). NKA (300nM) had no significant effect on either basal (2.3±0.3 Vs 2.1±0.3nM), nor stretch induced (6.2±1.5 Vs 5.8±1.1nM) ATP release (p≥0.05, n=6). Similarly, intravesical perfusion of NKA (300nM) had no significant effect on either basal (327±111 Vs 229±72nM, p≥0.05, n=6), or distension induced (355±129 Vs 247±77nM, p≥0.05, n=6) prostaglandin E_2 release (**fig 5.9.5**). However, in contrast, intravesical perfusion of NKA (300nM) significantly reduced ACh release at rest (0.33±0.08 Vs 0.07±0.03µM, p≤0.01, n=6), and during bladder distension (0.44±0.08 Vs 0.18±0.02µM, one-way ANOVA, Bonferroni multiple comparisons post-hoc test, **fig 5.9.4**)

This data implies that NKA, acting through NK2 receptors on urothelial cells is able to attenuate the release of ACh. To examine this further, calcium imaging experiments were performed on PMUCs. Application of NKA (300nM) to PMUCs caused a small but significant rise in intracellular calcium levels as reflected by an increase in fluorescent emissions over the course of sustained application (**fig 5.9.6**). The intracellular calcium response to NKA (300nM) was characterised by a slow increase

over the course of the response which did not return to baseline until washout. This effect was repeatable following wash-out of the agonist.

NKA (300nM) elicits a significant increase in intracellular calcium equal to 15.6±0.9% of maximal Ionomycin (5μM) response (**Fig 5.9.7**). This is equivalent to 61.7% of the maximum response to 100μM ATP.

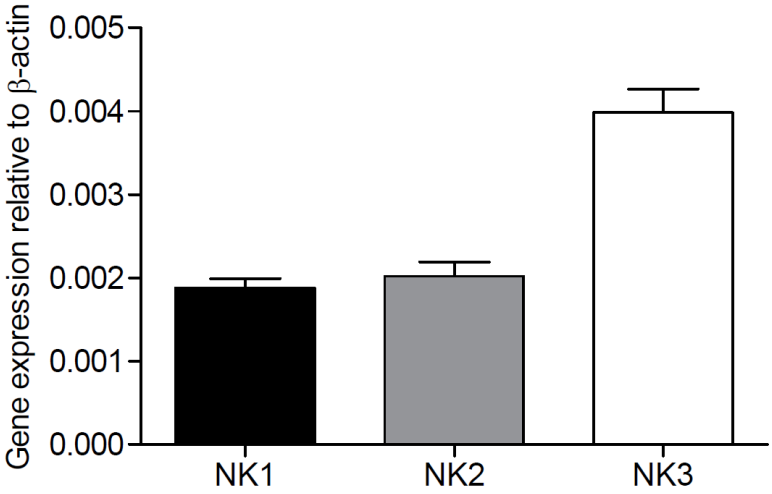

Fig 5.9.1 *Mean±(SEM) expression of tachykinin receptor mRNA relative to β-actin (ΔCT/CT) in PMUCs (n=3).*

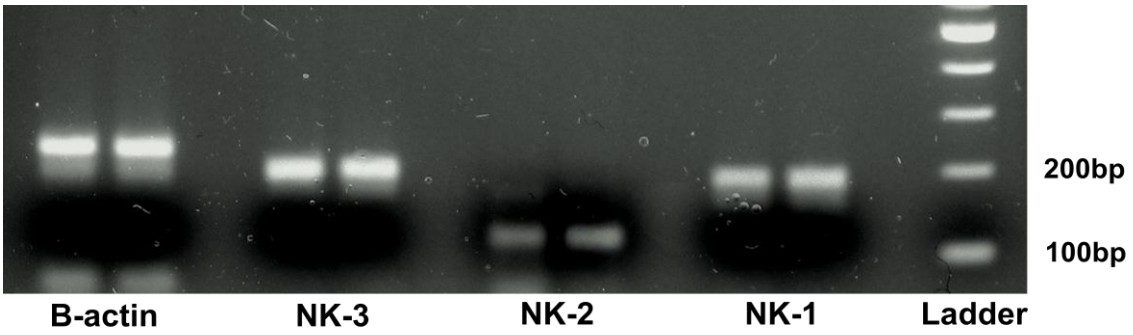

Fig 5.9.2 *Gel-electrophoresis of tachykinin receptor mRNA expression in PMUCs following qPCR. All three tachykinin receptors expressed a band at the appropriate product size.*

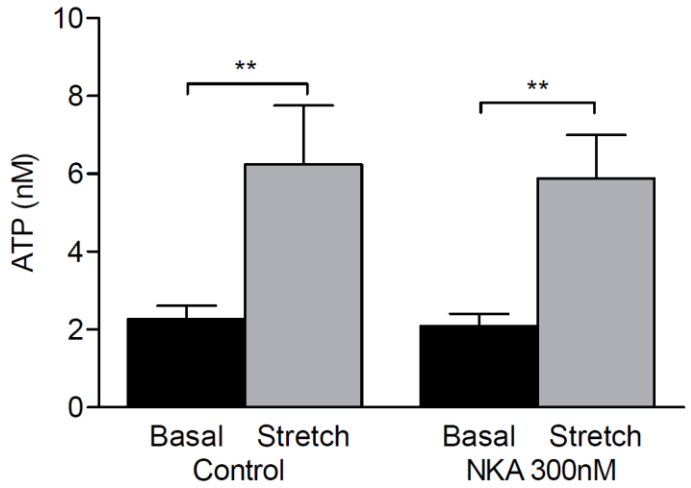

Fig 5.9.3 *Mean±(SEM) intraluminal ATP release from bladders at rest and at a maximum distension of 30mmHg with either saline or NKA (300nM), (**p≤0.01 Vs basal, n=6).*

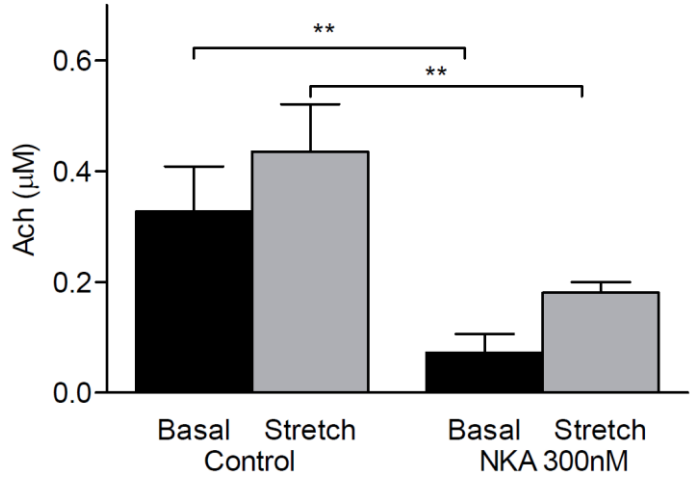

Fig 5.9.4 *Mean±(SEM) intraluminal acetylcholine release from bladders at rest and at a maximum distension of 30mmHg with either saline or NKA (300nM), (**p≤0.01 Vs control, n=6).*

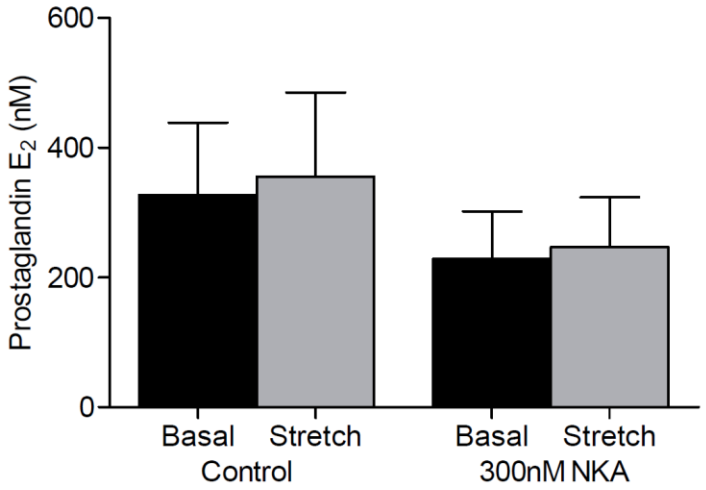

Fig 5.9.5 *Mean±(SEM) intraluminal prostaglandin E_2 release from bladders at rest and at a maximum distension of 30mmHg with either saline (n=6) or NKA (300nM, n=6).*

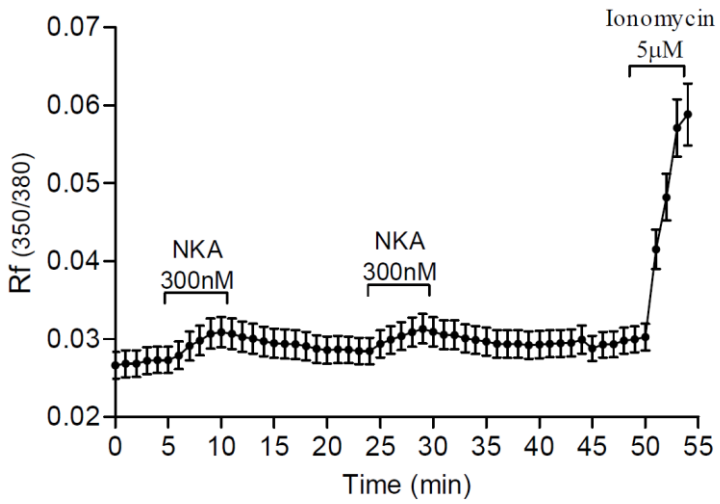

Fig 5.9.6 *Mean±(SEM) change in intracellular calcium levels of PMUCs to NKA (300nM, N=3, n-61).*

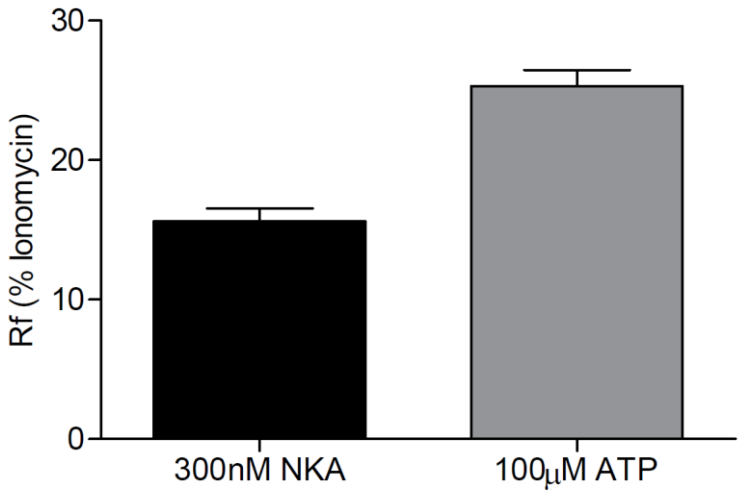

Fig 5.9.7 *Mean±(SEM) peak increase in intracellular calcium of PMUCs following application of NKA (300nM, N=3, n=61) and ATP (100μM, N=6, n=124).*

5.10 DISCUSSION

Tachykinin effects on detrusor smooth muscle and afferent firing

The first experiments conducted in this chapter were performed in order to determine the effect of tachykinins on detrusor smooth muscle contractions. Tachykinin agonists were chosen to identify the relative contribution of different tachykinin receptors to the response. Application of the NK2 receptor agonist NKA caused a significant increase in detrusor smooth muscle contraction which was characterised by a fast tonic component, followed by a slower phasic contraction component. These results are consistent with a large body of evidence stretching back 3 decades showing NK2-mediated effects on bladder contraction. NK2 receptors have been shown to be responsible for detrusor contraction in a number of species including hamster, rabbit, guinea pig, sheep, dog, pig and human (Longmore and Hill 1992; Giuliani, Patacchini et al. 1993; Guard, Pain et al. 1993; Burcher, Zeng et al. 2000; Rizzo and Hey 2000; Tucci, Bolle et al. 2001; Templeman, Sellers et al. 2003). The NK3 receptor agonist senktide had very little effect on detrusor contraction in these experiments and this is reflected in the literature, with NK3 receptors primarily confined to central mechanisms. Many other rodent studies, particularly in rat and guinea pig have shown an NK1 receptor component of detrusor contraction which is seen with both exogenous application of tachykinin agonists (Hall, Flowers et al. 1992; Longmore and Hill 1992) and with antagonism of NK1 receptors following capsaicin induced release of endogenous tachykinins from CSPANS (Maggi, Patacchini et al. 1991; Lecci, Giuliani et al. 1997). However, while substance P and senktide had small effects on contraction, this was significantly less than that seen with NKA. This could be due to either a lower affinity of these agonists at the NK2 receptor or a small contribution of NK1 and NK3 receptors to bladder contractile activity. To distinguish between these two possibilities would require a complete pharmacological assessment which was not the priority of the project. Confirming the role of the NK2 receptor in detrusor contraction, the selective NK2 antagonist GR159897 significantly attenuated NKA evoked contractions. The results observed suggest that, as with human and many other species, tachykinin induced contraction of detrusor smooth muscle in the mouse is mediated primarily by the NK2 receptor and provide evidence that a mouse model shares similarity to the human. NK2 receptor mediated detrusor muscle contractions were abolished by application of the L-type calcium channel blocker nifedipine. The sensitivity of the NK2 receptor mediated contractile response to nifedipine is consistent with Maggi et al's original findings in 1989. The dependence of detrusor smooth muscle on calcium induced calcium release from intracellular stores is well established (Quinn, Collins et al. 2004). Other groups have suggested a role for NK2 receptor

mediated IP_3 formation which may act upstream of calcium entry (Torrens, Beaujouan et al. 1995; Martin, Wheeler et al. 1997).

In association with tachykinin receptor mediated detrusor contraction, there was a significant increase in afferent sensory firing, consistent with the hypothesis that tachykinins are able to mediate both 'efferent' effects and afferent effects. It has been shown that stimulation of NK2 receptors is able to initiate micturition and the experiments in this thesis went further to investigate the effects of NKA mediated afferent nerve activity. It was found that bath application of NKA to a predistended bladder caused a significant increase in afferent nerve activity. Substance P and senktide were also able to stimulate a significant increase in afferent nerve firing but this was, as with detrusor contraction, significantly smaller than that of NKA. The ability of NKA to directly stimulate afferent nerves was further explored with the use of nifedipine. As mentioned above, nifedipine blocked NKA induced detrusor contraction, and this was accompanied by a complete abolishment of NKA induced afferent nerve firing which suggests that the afferent response to NKA is related to activation of mechanosensitive endings secondary to detrusor contraction. Only the role of the NK2 receptor was tested in this manner, but there was a consistent coordination with contraction and afferent activity with NK1 and NK3 receptor agonists substance P and senktide.

Previous studies have shown that tachykinin receptors, including NK1 and NK2 are able to mediate the excitability of DRG neurons including an increase in calcium currents and action potential threshold to various stimuli, (Sculptoreanu and de Groat 2007), and also an enhancement of capsaicin evoked currents (Zhang, Cang et al. 2007; Sculptoreanu, Artim et al. 2009). Activation of NK1, NK2, and NK3, receptors however, have also been shown to induce inward currents in rat DRG neurons and the lack of response seen in the whole bladder is somewhat of a mystery. (Yang, Yao et al. 2003)

As tachykinin receptors have been shown to mediate DRG neuron excitability, it was hypothesised that tachykinins may also be able to mediate the afferent nerves responsible for mechanosensitivity in the bladder which may be key to the contribution of tachykinins in stimulating micturition in a number of studies. To test this hypothesis, bath applied agonists were used to examine the role of tachykinins in the afferent nerve response to mechanical distension through bladder ramp distension and the actions of ATP on bladder afferents.

The effects of extraluminal tachykinin receptor agonists on bladder mechanosensitivity

It was necessary to further investigate the contribution of NK1, NK2 and NK3 receptor mechanisms on the interaction between detrusor muscle tone and afferent signalling by determining the effect of tachykinin agonists on the response to ramp distension, designed to mimic the effect of normal bladder filling.

Applied extraluminally, the NK3 receptor agonist senktide failed to modify either bladder compliance, as revealed from the pressure/volume relationship, or the afferent response to distension. Senktide has been previously shown to cause muscle contraction (Lecci and Maggi 2001), however, as described above, the small but significant effect of senktide in our own studies could have been via a non-NK3 receptor mechanism. Similarly, Lecci et al (2001) failed to determine if the response was mediated via actions on other tachykinin receptors. It is worth noting that Lecci et al found the contraction responses to senktide showed rapid desensitization. It remains possible that any effect of senktide in my studies could have been masked by receptor desensitisation, especially with the current perfusion protocol employed in which agonist concentration would rise gradually. Nonetheless, considering that senktide only initiated a small contraction and corresponding afferent response with a high concentration bolus dose in a predistended bladder, it is unlikely that the outcomes of these experiments would have been different if bolus doses were applied during ramp distension.

Substance P applied to the serosal side of the bladder preparation had no effect on either the compliance of the smooth muscle or afferent nerve discharge. These data support the concept that substance P does not play a significant role in mediating detrusor smooth muscle contraction in the mouse bladder. This is in contrast to other rodent models, including rat and guinea pig (Hall, Flowers et al. 1992; Longmore and Hill 1992) which have been shown to respond to NK1 agonists. However, these studies were fundamentally different, utilizing either isolated muscle strips (enabling the agonist to bind to all the layers of the bladder), or were applied intravenously/intraarterially in vivo, and via the vascular system, have access to the entire bladder. It is possible that other cells, possibly interstitial cells mediated NK1 agonist induced detrusor contraction in these studies.

In contrast to the lack of actions of bath applied substance P and senktide, NKA significantly decreased the compliance of the smooth muscle over the time course of the ramp distension. The increase in pressure at an equivalent volume is indicative of increased detrusor smooth muscle tone. This is consistent with the alterations in micturition patterns seen in a number of studies (Kibble 1996; Shaker, Tu et al. 1998) which were attributed to tachykinin actions. This is also consistent with

our finding that a bolus dose of NKA was able to cause significant contraction of the detrusor. What is noticeable though is that the sensory nerve discharge in response to ramp distension was not altered relative to the distension pressure, despite being achieved at lower volume. Furthermore, the afferent response to distension was not significantly changed following instillation of the NK2 receptor antagonist GR159897, again providing evidence that the NK2 receptor, and indeed the NK1 and NK3 receptors are not essential in normal peripheral sensation of bladder volume. Previous evidence has shown that it is possible to increase mechanosensitivity to distension via systemic application of tachykinins in vivo (Kibble 1996) and that that antagonism of the NK2 receptor reduced mechanosensitivity (Kibble 1996). However, in such circumstances it is possible that the tachykinin under investigation may have had a sensitising effect on central micturition processes independent of a direct peripheral role on bladder sensory afferents. This is suggested by experiments that show there is a central action of tachykinins in the spinal cord which are able to modulate the micturition reflex (Mersdorf, Schmidt et al. 1992; Ishizuka, Igawa et al. 1994; Lecci, Giuliani et al. 1994; Lecci and Maggi 2001) via sympathetic and parasympathetic outflow. There is some evidence however, that NKA is able to sensitise calcium channels in peripheral sensory neurons (Sculptoreanu and de Groat 2003), as well as enhancing excitability in capsaicin-responsive DRG neurons (Sculptoreanu and de Groat 2007). In this respect it is perhaps surprising that exogenous NKA did not potentiate the mechanosensitive peripheral sensory neurons in the current study. However, these studies on isolated DRG neurons did not select on the basis of bladder projection, nor were they able to assess the impact on mechanosensitive ion channels and thus interpretation is difficult based on the current observations.

The NKA induced decrease in bladder compliance was completely attenuated by GR159897 which, when applied alone, also significantly increased bladder compliance in normal ramp distensions. These results suggest that there is a basal bladder tone during distension, and that endogenous tachykinins acting through NK2 receptors could contribute to this, either directly at the level of the muscle, or a possible secondary mechanism via the urothelium or interstitial cells. This is supported by a previous study showing that an NK2 antagonist was able to cause relaxation of the basal muscle tone following distension to a set volume (Lecci, Giuliani et al. 1998) and indirectly via depletion of neuropeptides (Lasanen, Tammela et al. 1992). It is possible that during bladder distension, mechanosensitive afferents innervating the detrusor smooth muscle are stimulated to release neuropeptides such as NKA, which are in turn able to act on the muscle (Morrison 1999). The difference in the compliance curve was evident by the time the bladder pressure had reached 10-15mmHg which represents a physiological level of distension during the more compliant part of the pressure/volume curve. This would imply that low threshold afferents are activated at this stage and

not higher threshold nociceptive fibers, contradicting the general view that the neuropeptide containing C-fiber afferents are activated only at high threshold. However, in rats, greater than 70% of bladder DRG neurons appear to contain multiple neuropeptides (Fowler, Griffiths et al. 2008). In the rat there is also a greater proportion of unmyelinated C-fiber (TRPV1 expressing) bladder afferents than Aδ-fiber afferents (Vera and Nadelhaft 1990; Andersson 2002), and these fibers have been shown to respond to slow distension (Morrison 1999) as well as low bladder pressures (Fowler, Griffiths et al. 2008).

It is hard to interpret a direct role of tachykinins on mechanosensitivity from these results because not only are stretch sensitive afferents activated by the process of bladder distension but tachykinins are also able to induce a direct bladder contraction and a change in compliance, and this can make interpretation of the cause of these responses difficult.

The concept of NKA induced potentiation of afferent nerve activity and detrusor smooth muscle contraction was tested further by examining the interaction with ATP. These experiments examined P2X sensitive afferents, which have been proposed to be important in mechanosensation of the bladder via urothelial release of ATP (Vlaskovska, Kasakov et al. 2001; Cockayne, Dunn et al. 2005). αβMe-ATP induced afferent firing was unchanged by a sub-threshold dose of NKA, suggesting that low doses of NKA are not able to sensitise afferent nerves to purinergic stimulation. However, the purinergic component of detrusor contraction was significantly potentiated. The mechanism underlying this potentiation is unclear but liberation of intracellular calcium and sensitisation of second messenger pathways is a possibility. This theory is in part supported by experiments in which there was an increase in ATP induced calcium mobilisation in detrusor smooth muscle from aged animals, associated with an enhanced purinergic mediated contractions (Gomez-Pinilla, Pozo et al. 2011). In theory these cellular modifications could occur as a result of increased tachykinin release from CSPANS. It is also proposed that a subthreshold dose of NKA mimics the enhancement of tachykinin immunoreactivity seen in disease/inflammation (Moore K H 1992; Callsen-Cencic and Mense 1997; Smet, Moore et al. 1997; Schnegelsberg, Sun et al. 2010), and considered responsible for the increase in the atropine insensitive component of detrusor contraction observed in humans with bladder pathologies (Yoshida, Homma et al. 2001). This theory is consistent with an 'efferent' function of tachykinins, and an upregulation of tachykinin sensitive nerves in animal models of chronic inflammation and idiopathic detrusor overactivity in humans (Moore K H 1992; Callsen-Cencic and Mense 1997; Smet, Moore et al. 1997). There have been no previous studies examining the specific effect of tachykinins on the purinergic component of contraction but EFS studies have generally concluded that although tachykinins may have a role in the NANC component of

contraction, it is not associated with the purinergic component (Meini and Maggi 1994; Azadzoi, Radisavljevic et al. 2008). However, the focus of these studies was antagonism of contraction rather than its potentiation.

The effects of Intravesical tachykinin receptor agonists on detrusor smooth muscle and afferent nerve activity

As alluded to above, application of tachykinin agonists to the serosal side of the bladder and the effects it may have on mechanosensitivity may be masked by contraction of the detrusor by direct actions of NK2 receptors. In order to try and remove the direct detrusor contraction component, tachykinins were also applied intravesically as this approach has previously been able to stimulate micturition reflexes.

Neither intravesical senktide nor substance P had any effect on either detrusor smooth muscle compliance, or afferent nerve discharge. These results are consistent with previous experiments in rats which failed to identify a response to NKB (Ishizuka, Mattiasson et al. 1995). It is interesting however, that substance P failed to stimulate changes in compliance or afferent nerve activity. These results add to the increasing evidence that the NK1 receptor is not essential for mediating the peripheral tachykinin responses in the bladder and that NK1 receptors on sub-urothelial afferent nerves are not activated by intravesical substance P. Intravesical instillation of substance P in rats failed to stimulate micturition (Ishizuka, Mattiasson et al. 1995) corroborating the current results.

Intravesical perfusion of the NK2 receptor agonist NKA induced a significant decrease in smooth muscle compliance which was blocked by GR159897, with the bladder exhibiting significant increases in intravesical pressure at much lower volumes, yet the afferent nerve response to distension remained unchanged. Although there was no overall variation in afferent nerve activity in relation to an increase in intravesical pressure, a significant change in transient afferent nerve activity was observed with a potentiation of phasic detrusor contractions during distension.

A maximal pressure of 30mmHg was used in these experiments to ensure reproducibility of spontaneous activity during ramp distension, which was not always observed at faster filling rates and higher pressures. It has been reported previously (Maggi, Giuliani et al. 1991; Ishizuka, Mattiasson et al. 1995) that in an in vitro model of guinea-pig whole bladder, intravesical instillation of an NK2 agonist facilitated the occurrence of rhythmic contractile activity. Spontaneous contractile activity was potentiated in the current experiments also, but went further to examine the associated

afferent nerve discharge. Both the magnitude and the frequency of spontaneous detrusor activity were significantly increased with intravesical NKA and the effect was deemed specific to the NK2 receptor since it was completely attenuated by GR159897. Neither senktide, nor substance P had any effect on spontaneous detrusor activity. A striking feature of the current experiments was the concurrent bursts of afferent nerve activity that were coupled to the detrusor contraction which were significantly greater than control and were more apparent at low/more physiological levels of bladder pressure. It has been shown that sensory afferent nerve activity is an essential stimulus for micturition, but the significance of phasic afferent activity such as this has yet to be proven. However, a correlation between small detrusor contractions termed micromotions and increased sensation has been observed in human patients with increased bladder sensation during the filling phase of cystometry (Drake, Harvey et al. 2005) and a number of studies have reported increased non-voiding contractions in animal models of cystitis induced bladder hyporeflexia (Charrua, Cruz et al. 2007; Wang, Wang et al. 2008). The firing of afferent nerves associated with these spontaneous detrusor contractions at low levels is equivalent to distension induced afferent firing occurring at much greater bladder pressures under normal ramp distension. It is thus hypothesised, that this phasic bursting of sensory afferents is an adequate stimulus to facilitate the micturition reflex observed in previous experiments which did not record afferent activity, and these results provide a mechanism whereby increases in tachykinin levels can result in the development of OAB and bladder pain symptoms. Spontaneous contractions which increase the pressure in the bladder up to the point at which afferent firing generates physiological sensations to void would occur at a much reduced volume (Igawa, Zhang et al. 2004).

These results indicate two things, firstly, that NKA, acting on NK2 receptors upon the urothelium is able to modulate detrusor smooth muscle compliance, and secondly, that the actions of intravesical NKA, and the direct downstream effects exclude actions on the sensory afferent nerves in the normal bladder as there is no direct effect of NKA on mechanosensitivity. It could be considered that NKA is able to penetrate the urothelium and act directly on the detrusor smooth muscle, however, previous studies using a similar methodology in both rats and guinea pigs, where detrusor contraction is mediated by both NK1 and NK2 (Hall, Flowers et al. 1992; Longmore and Hill 1992), found only NKA can stimulate micturition when given intravesically (Maggi, Giuliani et al. 1991; Ishizuka, Mattiasson et al. 1995), and only an NK2 selective antagonist can attenuate this response. These results pertain to the earlier suggestion, that intravesical NKA stimulates micturition, a decrease in bladder compliance, bladder capacity and residual volume, and rhythmic contractile activity via a different method to serosal application or intra-arterial/intravenous application of tachykinin agonists, and this route is most likely via stimulation of the urothelium. Another point of

interest is that intravesical instillation of the NK2 antagonist blocked NKA responses but the antagonist alone did not have an effect on either phasic detrusor contraction or afferent nerve activity. It is possible that an endogenous role for tachykinins via this pathway is limited under physiological conditions but could become more influential during disease, especially those involving inflammation affecting the urothelial layer.

Further evidence of a role for the urothelium in mediating the alterations in detrusor muscle contraction and subsequent sensory discharge was tested by investigating the ability of NKA to influence the release of urothelial mediators, and the ability of intravesical tachykinins to induce changes in afferent nerve activity whilst the bladder was at rest.

Firstly, intravesical infusion of all three tachykinin agonists, NKA, substance P, and senktide were without effect on baseline afferent activity of the bladder. The results again show that there is a no direct effect of tachykinins on sensory afferent nerves in the suburothelium. These experiments nonetheless provide an insight into the action of tachykinins within the bladder, and how they might influence detrusor smooth muscle contraction. The urothelium of the bladder, aside from its barrier function has an essential role as a mechanosensor, releasing in response to stretch, a number of neurotransmitters, including but not limited to acetylcholine, ATP, prostaglandins, peptides, nitric oxide, NGF, and cytokines. It is possible, that the effects seen by intravesical instillation of tachykinins occur as a result of changes to distension induced mediator release, and are therefore only seen during ramp distension of the bladder.

The urothelium of the bladder has many proposed functions, but its primary and most established role is as a barrier to the contents of urine. It could be loosely proposed, therefore, that instillation of an agonist into the bladder lumen will interact only with the cells in the immediate vicinity of the urothelium, and that any other effects seen, such as those observed with changes in bladder compliance and the amplification of spontaneous activity and afferent nerve activity, will be downstream of urothelial activation. Measurements of ATP, acetylcholine and PGE_2 were made before bladder distension and following ramp distension at 30µl/min to a maximal pressure of 30mmHg. Consistent with previous literature (Birder, Nakamura et al. 2002; Hanna-Mitchell, Beckel et al. 2007; Sadananda, Shang et al. 2009; Tanaka, Nagase et al. 2011) it was found that the bladder has a tonic release of acetylcholine and ATP, as well as PGE_2. It was also shown that ramp bladder distension induces a significant increase in both ATP and acetylcholine, but not PGE_2. Intravesical instillation of NKA had no effect on ATP or PGE_2 release at rest or during bladder distension, but acetylcholine release was significantly reduced during basal and also distension conditions yet there was no effect on the change in the ability of stretch to release acetylcholine. It has been previously

shown that NKA has no effect on ATP release from urothelial cells (Cheng, Mansfield et al. 2011) but this is the first study to investigate the effects of other mediators.

There is a network of interstitial cells identified within the bladder which have been proposed to be intermediates in sensory transduction pathways. These cells have been shown to mediate spontaneous detrusor contraction (Biers, Reynard et al. 2006) through gap junctions (Ikeda, Fry et al. 2007), and application of Imatinib or other tyrosine kinase inhibitors has been shown to reduce spontaneous detrusor contractions (Biers, Reynard et al. 2006). It is therefore proposed that the actions of NKA on urothelial cells, and their subsequent alterations in mediator release alter the functional dynamics of the interstitial cells and thus the underlying detrusor smooth muscle. Interstitial cells have been shown to express purinergic (Sui, Wu et al. 2006; Li, Xue et al. 2013) and muscarinic receptors (Johnston, Carson et al. 2008) and generate large depolarizing responses to ATP (Wu, Sui et al. 2004). An important role for P2Y receptors has been shown (Sui, Wu et al. 2006; Fry, Young et al. 2012) and thus a role in processing ATP-mediated sensory activation (Liu, Takahashi et al. 2009) is also a possibility. It could be possible that with multiple inputs to interstitial cells from mediators released from the urothelium, that downregulation of the acetylcholine/muscarinic component leaves only purinergic excitation which may mediate a phenotypic switch in spontaneous detrusor contractions. Indeed, in overactive bladder there is an increase in interstitial cell number in both humans and rats (Ikeda, Fry et al. 2007; Roosen, Datta et al. 2009) which is also associated with an increase in ATP release from the urothelium, and as mentioned previously, an increase in tachykinin immunoreactivity in CSPANS which are known to innervate the urothelial layers. An intact urothelium has also been shown to be necessary for normal interstitial cell function (Ikeda and Kanai 2008), whilst interstitial cells were augmented by ATP (Sui, Wu et al. 2008) and which lead the author to suggest that intrinsic (spontaneous) activity of the bladder is of urothelial origin (Ikeda and Kanai 2008).

Urothelially mediated responses

In order to test the hypothesis that intraluminal NKA is able to act on urothelial cells, it was necessary to show that tachykinin receptors are not only present upon urothelial cells, but are also functional.

NK1, NK2 and NK3 receptor mRNA was present within PMUCs, providing some credence to the theory that NKA is able to act on urothelial cells. Although it is not direct evidence for tachykinin receptor expression, the presence of all three tachykinin mRNAs suggests that the framework is

there for the cells to express these receptors and it could be the case that these receptors are more highly expressed during inflammation and disease, just as is seen in afferent sensory neuron immunoreactivity. Particularly intriguing is the high levels of NK3 receptor mRNA yet studies have not found a role for this receptor in the periphery. Despite the more prolific expression of the NK3 receptor within the urothelium, the NK2 receptor was focussed on as evidence up to this point suggested that NK2 was more functionally relevant.

The final experiments performed confirmed that functional NK2 receptors exist on the urothelium, and through calcium imaging of PMUCs it was shown that they respond to repeated application of NKA with a sustained and repeatable calcium response. These results also confirm the hypothesis that intravesical NKA is able to stimulate NK2 receptors on urothelial cells and this could be the initial stage in a process in which urothelial activation by tachykinins contributes to the maintenance of the micturition reflex through changes in mediator release and interactions with the underlying structures of the bladder wall, including interstitial cells, detrusor smooth muscle and afferent nerves. Other studies have previously alluded to the presence of tachykinin receptors on urothelial cells, but these results are the first to confirm that there are functional NK2 receptors upon the urothelium and their response could mediate downstream mechanosensation.

Conclusion

The role of tachykinins in the peripheral control of mouse bladder urothelium, detrusor smooth muscle, and afferent sensory nerves have been outlined above. This study agrees with previous findings suggesting that the NK1 and NK3 receptors have little function within the normal mouse bladder and that the NK2 receptor is shown to have a role in smooth muscle contraction.

This study has gone further than previous reports by employing various techniques in an attempt to determine the precise mechanosensitive role of tachykinins within the bladder. It is the first study of its kind to link previous functional evidence of increased bladder contractions and micturition by examining the role of tachykinins on afferent nerve excitability in the mouse, particularly in response to physiological bladder distension. This study is also the first to show that functional NK2 receptors exist on the urothelium and that through agonist binding, they are able to alter the afferent sensations associated with the micturition reflex.

The importance of these results in normal bladder function have been shown to be limited, however, there is significant potential for tachykinin mediated responses to play an essential role in bladder

disease states. Thus far it has been shown that there is increased tachykinin immunoreactivity and increased CSPAN immunoreactivity in bladder disease providing a mechanism whereby additional tachykinins can be released from the peripheral endings in the vicinity of the urothelium and interstitial cells and therefore has the potential to modulate afferent excitability.

It would also be interesting to investigate if tachykinin receptor expression increases with inflammation and cystitis in the urothelium, as it has been observed that NK2 receptors in the urothelium respond to tachykinins and this activation can lead to potentiation of rhythmic contractions and afferent nerve activity, and could provide a novel route in which to target bladder symptoms.

CHAPTER 6: GENERAL DISCUSSION

This thesis describes a body of work investigating various pathways and receptors involved in bladder mechanosensitivity using a variety of techniques which converge on the hypothesis that sensory afferent function is a key modulator of the micturition reflex in both health and disease. The importance of sensory pathways in governing the micturition reflex has highlighted them as a target for the treatment of overactive bladder and interstitial cystitis.

Overactive bladder syndrome (OAB) is defined by the ICS committee as 'urgency, with or without urge incontinence, usually with frequency and nocturia (Abrams, Cardozo et al. 2002) and is believed to affect approximately 16-17% of the adult population (Milsom, Abrams et al. 2001) with a number of contributing risk factors including age, child birth, and increased BMI (Rortveit, Daltveit et al. 2003; Maserejian, Minassian et al. 2014),

The aetiology of OAB is still unknown but is considered to be multifactoral. A major concept now under intense investigation is that of abnormal afferent firing from the bladder wall. This is based on the observations that urge and frequency of micturition are symptoms associated with the filling phase of the micturition reflex rather than during voiding. This has been considered in respect to increased spontaneous activity of the detrusor smooth muscle (Andersson 2010) upregulation of C-fiber afferent activity and subsequent plasticity (Ouslander 2004), as well as increased activation of sensory C-fiber afferents in the sub-urothelium. There is also a growing consensus that release of a number of chemical mediators, in particular ATP, from non neuronal sources such as the urothelium and acting on the afferent nerves innervating both the urothelium and sub-urothelium is a substantial contributing factor to OAB symptoms.

Understanding the complex sensory mechanisms which initiate the micturition reflex could provide novel targets for the development of more effective pharmacotherapy for bladder disorders. The aim of this work was to directly investigate the mechanisms underlying mechanosensation within the bladder using an in vitro mouse model that enables the recording of afferent nerve activity and intravesical pressure together with cell based assays using primary cultures of pelvic DRG and urothelial cells. This final discussion will focus on the control of mechanosensation and how the results found in this thesis contribute to our overall understanding of this process.

Mechanosensitivity

Mechanosensitivity, the process by which sensory afferent nerves convey information regarding the state of bladder filling, is a crucial determinant in the initiation of the micturition reflex. Relaxation of the bladder during the storage/filling phase of micturition is required to maintain low intravesical pressure. Sensory afferent firing from the bladder is coordinated in the spinal cord and stimulates inhibitory sympathetic input to the detrusor smooth muscle causing relaxation, as well as an inhibitory effect on bladder ganglia to inhibit excitatory parasympathetic inputs to the bladder. The switch from urine storage to voiding occurs when sensory afferent activity reaches a critical threshold, and central mechanisms cause a switch in the autonomic outflow resulting in parasympathetic excitation and sympathetic inhibition. Despite this, the current pharmaceuticals for OAB are designed to modulate cholinergic pathways with a view to suppressing parasympathetic drive, as opposed to targeting the sensory process mediating this response.

What remains unclear however, is the mechanisms that regulate the sensory afferent discharge from the bladder, and most importantly, determining how this can be effectively targeted to improve the urgency, frequency and pain symptoms of OAB and IC.

The detection of bladder stimuli by afferent nerves is inherently linked to the feelings which progress from an urge to void, followed by discomfort, and finally pain associated with an abnormally full bladder. Normal bladder function relies on several different afferent subtypes, in different layers of the bladder wall and following different pathways to the spinal cord.

There are typically considered to be two structurally distinct populations of bladder afferents; those with terminal receptive fields in the detrusor smooth muscle, and those in the vicinity of the urothelium (Zagorodnyuk, Brookes et al. 2010). The structural location of these two populations of afferents reflects the two current theories for how mechanosensation is triggered. The first theory is that initiation of afferent firing is caused by mechanical deformation of the urothelial layer and detrusor smooth muscle. This type of mechanosensitivity is dependent on the distortion of mechanically gated ion channels on peripheral varicosities innervating both the muscle layer and the sub-urothelial layer (Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). These respond to either muscle stretch and act as in-series tension receptors or to distortion of the urothelium by experimental stoking. The second theory is that of an indirect mechanosensation mechanism through the release of mediators from the urothelium which either directly activate sub-urothelial afferents or have a modulating influence over their mechanosensitivity. This latter concept suggests that the urothelium acts as a sensory web, a notion that has recently been gaining popularity.

Bladder distension evokes the release of a number of mediators that act on chemosensitive afferent endings which express a number of receptors that trigger changes in excitability, for example TRP channels and purinergic receptors (Rong, Spyer et al. 2002; Zagorodnyuk, Costa et al. 2006; Daly, Rong et al. 2007; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). There is, of course, likely to be a contribution of both these pathways in the control of reflex micturition and a blurring between the classifications of afferents, particularly in disease, where there is sensitisation of mechanosensitive afferents, and the recruitment of silent afferents that enhance the sensory signals flowing from the bladder towards the CNS (Häbler, Jänig et al. 1990; Shea, Cai et al. 2000; Rong, Spyer et al. 2002; Xu and Gebhart 2008).

These two concepts provide a simplistic view of mechanosensation in the bladder as can be seen in ***fig 6.1.2***. The role of the urothelium is becoming increasingly apparent, as both a transducer of tension and in the release of neurotransmitters from the distending bladder, and as such is currently considered a major sensory structure. However, other cells in the bladder wall have been proposed to contribute to mechanosensitivity, particularly interstitial cells, which may act as intermediaries responding to mediators released from the urothelium and have a modulating influence over smooth muscle contractility and afferent excitability (Wiseman, Fowler et al. 2003; Davidson and McCloskey 2005; Fry, Sui et al. 2007)

A major focus of this thesis has been to investigate these mechanosensitive mechanisms and attempt to distinguish the actions of the urothelium from those of the afferent nerves and detrusor smooth muscle in order to provide a clearer picture of the control of bladder sensation.

Mechanosensation mediated by stretch/tension

Increased tension on the detrusor smooth muscle either via mechanical stretch or active contractions that causes an increase in intravesical pressure has been shown in this thesis to be a major stimulus of sensory nerve activity. However, the precise relationship between the muscle and afferent ending is difficult to determine because of the complex network of internal structures contributing to a multifaceted afferent response.

Afferent nerve recording experiments performed in this thesis employed ramp distension of the bladder to better mimic the slow filling of the bladder and accompanying rise in intraluminal pressure that occurs physiologically. It is observed that afferent nerve activity is inextricably linked to changes in intraluminal pressure. This is similar to other studies inducing bladder distension in vitro

(Vlaskovska, Kasakov et al. 2001; Rong, Spyer et al. 2002; Daly, Rong et al. 2007) as well as bladder stretch in vitro (Zagorodnyuk, Costa et al. 2006; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008) where a distinction between low and high threshold afferents has been made. In those studies, a larger proportion of the afferent response was attributed to low rather than high threshold mechanoreceptors, particularly within the physiological ranges of distension (Igawa, Zhang et al. 2004).

An important consideration in this thesis was the relationship between afferent firing and bladder compliance which became even more apparent upon comparing the two bladder filling rates used in this thesis, 30µl/min and 100µl/min. As described in chapter 4, an infusion rate of 100µl/min has been used in a number of previous studies (Vlaskovska, Kasakov et al. 2001; Rong, Spyer et al. 2002; Cockayne, Dunn et al. 2005; Daly, Rong et al. 2007) investigating afferent nerve responses to bladder distension and has been considered as a standard for these experiments. The effects of tachykinins were studied using a slower fill rate of 30µl/min in order to maintain consistent phasic detrusor contractions. Further analysis of bladder distension at the two filling rates from all experiments revealed a significant difference in the compliance and afferent response profiles ***(fig 6.1.1)***. A significant increase in the compliance of the muscle is observed with a slower filling rate, allowing a larger volume before reaching a set pressure and a reduction in the afferent response to given pressures. These results support the idea of in-series tension receptors within the bladder and that at a slower rate of filling, the structures have the ability to accommodate the volume more effectively and less tension is produced in the bladder wall.

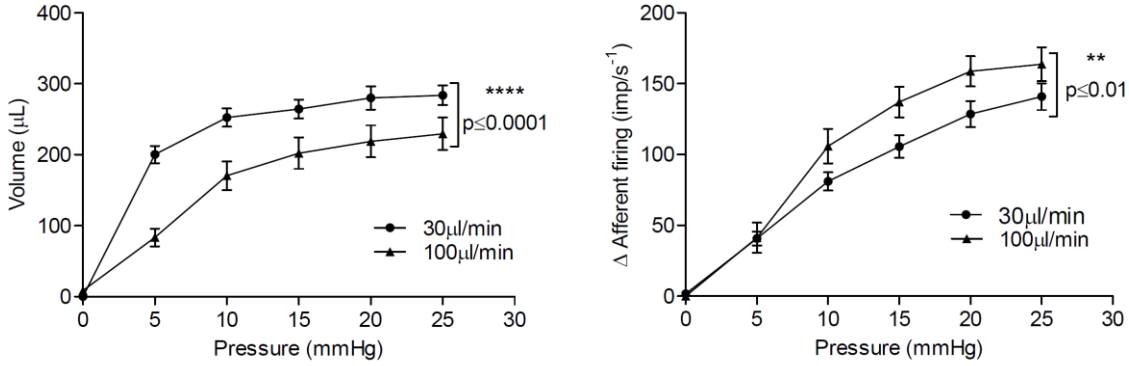

Fig 6.1.1 *Mean±(SEM) pressure/volume and pressure/nerve relationships of bladders distended with saline at either 30µl/min (n=50) and 100µl/min (n=40).*

An infusion rate of 30µl/min is considered to be more within the physiological range of mouse bladder distension and rates between 20-30µl are commonly used during cystometry recordings in

mice (Andersson 2011). It would be my recommendation that future studies examining physiological bladder function be performed at reduced infusion rates, to ensure the bladder is allowed to accommodate the increase in volume without unphysiological levels of stretch in the bladder wall. It is possible that a faster infusion rate provides information regarding nociceptive afferent firing from the bladder.

The role of tension receptors and their ability to mediate afferent nerve excitability was especially apparent with the spontaneous detrusor contractions that occur during ramp distension. Even small changes in intravesical pressure directly coupled to detrusor contraction, in the range of 1-2mmHg, were able to induce large transient increases in afferent nerve activity. These contractions were of myogenic origin since they persisted in the presence of tetrodotoxin (***fig 2.6.4)***. Phasic detrusor activity in whole bladder preparations has previously been shown in the guinea pig, and it was revealed, using a novel electrode displacement technique, that these phasic detrusor contractions were associated with localised stretch of the bladder wall (Drake, Harvey et al. 2003; Drake, Harvey et al. 2005). If the detrusor is overactive and is producing large spontaneous contractions and localised stretch of the bladder wall, this would result in an exaggerated sensory afferent discharge from the periphery, and rapidly reach the threshold for micturition at lower bladder volumes thus causing symptoms such as urge and discomfort. This is a potential mechanism underlying increases in micturition frequency in animal models of bladder cystitis which are associated with increases in the frequency of reflex (non-voiding) bladder contractions (Charrua, Cruz et al. 2007; Wang, Wang et al. 2008). Furthermore, a greater prevalence of phasic detrusor activity has been correlated to patients with increased sensation during filling cystometry (Drake, Harvey et al. 2005). Despite the phenotype in this case being increased contractions of the detrusor, the trigger for this could potentially arise from the urothelium as was shown in the experiments employing intravesical instillation of NKA, and could include a contribution from interstitial cells. Indeed, it has been shown that removal of the urothelium significantly inhibits stretch sensitive muscle/urothelial afferents (Zagorodnyuk, Gibbins et al. 2007) and these endings are also able to respond to ATP.

The data are in agreement with a considerable body of literature showing that a major proportion of bladder pelvic nerve mechanosensation occurs via the muscular and muscular/urothelial stretch sensitive afferents in the mouse, rat, guinea pig and cat (Häbler, Jänig et al. 1993; Sengupta and Gebhart 1994; Su, Sengupta et al. 1997; Shea, Cai et al. 2000; Zagorodnyuk, Costa et al. 2006; Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). This was further explored using a number of pharmacological tools found to stimulate sensory afferent firing which were directly coupled with

contraction of the muscle. This was most apparent using NKA, which via NK2 receptors induced afferent activity that was abolished following elimination of the contractile response.

In contrast, P2X receptor stimulation of afferent nerves with αβMe-ATP evoked a significant increase in afferent nerve discharge even in the absence of muscle contraction. In the presence of calcium, αβMe-ATP induced a sharp contraction of smooth muscle and an even greater afferent nerve discharge than that in calcium free medium. This suggests that there are two distinct pathways responding to P2X receptor stimulation, a direct activation of P2X receptors on sensory afferent endings, and an indirect afferent response as a result of P2X dependent contraction of detrusor smooth muscle and the subsequent activation of mechanically gated afferents.

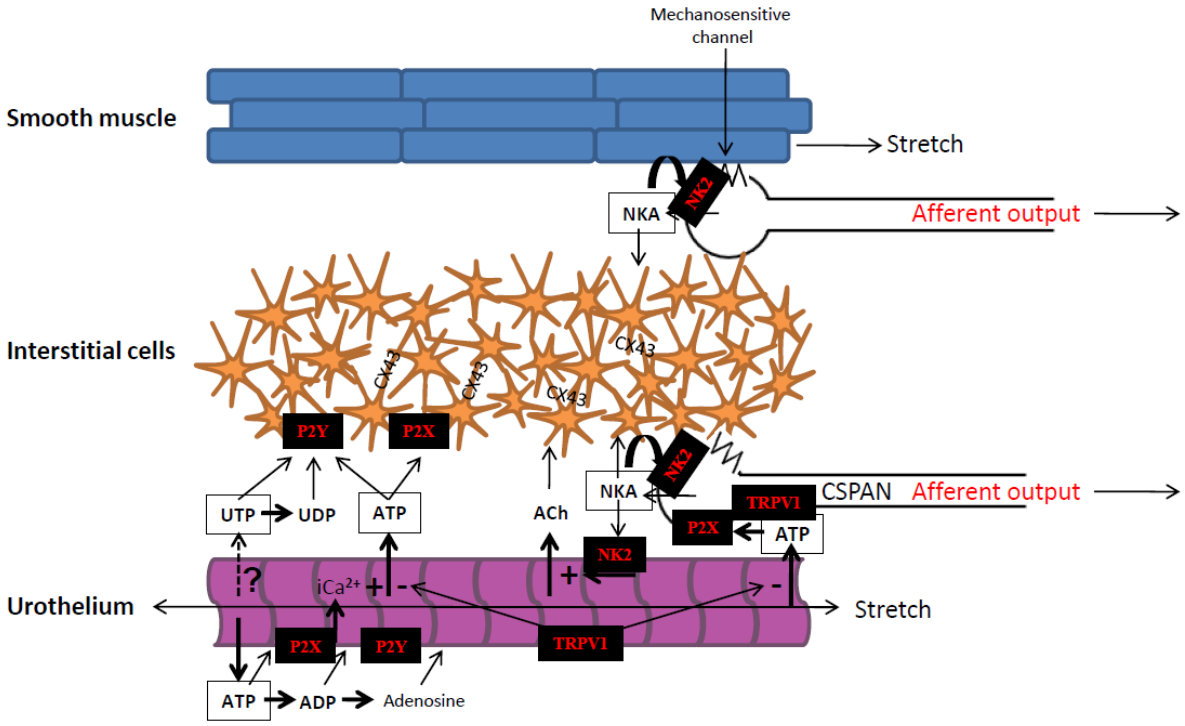

Fig 6.1.2 *Theoretical model depicting interactions between urothelial cells, interstitial cells, and detrusor smooth muscle during bladder distension, and the control of afferent nerve firing. Stretch of urothelial cells can release mediators that target bladder nerves and interstitial cells; urothelial cells can also be targets for neurotransmitters released from suburothelial nerves or through autocrine mechanisms. Afferent nerves can be activated by direct or indirect mechanotransduction. Abbreviations: Ach, acetycholine; ATP, adenosine triphosphate; ADP, adenosine diphospahte; CX43, connexion 43 gap junction protein; CSPAN, capsaicin sensitive primary afferent neuron; iCa^{2+}, intracellular calcium; NKA, neurokinin A; NK2, tachykinin 2 receptor; P2X and P2Y, purinergic receptors; TRPV1, transient receptor potential vanilloid 1; UTP, uridine triphosphate; UDP, uridine diphoshate.*

Mechanosensation not mediated by stretch/tension

A number of studies have identified a component of the afferent nerve response that is not a result of bladder stretch/tension. Xu and Gebhart (2008) identified a proportion of pelvic afferents that did not respond to stretch and had virtually no response to stroking or probing, which they called urothelial afferents. A similar subclass of afferents was reported to be present in the guinea pig bladder (Zagorodnyuk, Gibbins et al. 2007) which were proposed to act as chemoreceptors. These afferents showed reduced responses following removal of the urothelium, and this was proposed to be as a consequence of removing ATP release from the urothelium. However, these studies did not assess the role of these afferents in response to the physiological stimuli of bladder filling. It is these urothelial afferent fibers which are believed to be responsible for the functionally distinct sensitivity to αβMe-ATP and capsaicin which are reported to make up approximately 10% of the total afferent innervations (Zagorodnyuk, Gibbins et al. 2007; Xu and Gebhart 2008). The TRPV1 receptor is not considered to be mechanically gated. Nevertheless, as discussed in chapter 4, the afferent response to ramp distension was attenuated in the $TRPV1^{-/-}$ (KO) mice, and was proposed to arise from these urothelial endings. Further evidence for this comes from the observation that afferent responses to αβMe-ATP were significantly attenuated both in the $TRPV1^{-/-}$ (KO) mice, and following pharmacological antagonism of TRPV1, yet the amplitude of the detrusor muscle contraction was unchanged. This is evidence that the two distinct P2X receptor pathways described above (direct and secondary to contraction) can operate independently of each other. TRPV1 receptor function appears not to be related to direct mechanosensitivity induced by muscle contraction, but is coupled to the afferent mechanisms responsible for αβMe-ATP induced afferent sensitivity that is independent of muscle contraction. This is further indicated by the observation that in $TRPV1^{-/-}$ (KO) mice there was no change in bladder compliance during distension but afferent nerve firing was significantly attenuated. This again is evidence that these two mechanosensitive pathways can function independently, although the relative contribution of this urothelial component of mechanosensation appears much smaller than that from the muscle.

Further evidence for this distinct purinergic based afferent response that is independent of detrusor contraction can be found from previous studies using P2X knockout mice, although the contribution of the urothelial component is hard to quantify given the way the data is presented (Vlaskovska, Kasakov et al. 2001). This is because while afferent activity was significantly attenuated in the $P2X_3^{-/-}$ (KO) mice, this was accompanied by a significant change in muscle function (Vlaskovska, Kasakov et al. 2001). The volume required to elicit maximum pressure was almost double that seen in the WT mouse, with a corresponding increase in the time taken to achieve this pressure, thus indicating that

the compliance of the smooth muscle had changed, and with it, the level of afferent firing. Significant changes in bladder volumes as well as decreases in non-voiding contractions were observed in another study using $P2X_3^{-/-}$ (KO) mice (Cockayne, Hamilton et al. 2000) which also found no significant change in the pressure required to elicit micturition. This tends to confirm the view that pressure or bladder wall tension is a major stimulus for afferent evoked micturition.

Further studies by Cockayne et al (2005) compared directly the relationship between afferent nerve firing and intravesical pressure in single and double knockouts of $P2X_2$ and $P2X_3$ receptors since these receptors often form heteromeric assemblies (North 2002). A significant change in detrusor muscle function was observed, but analysis of the afferent nerve/pressure response curves indicated that whole nerve and single unit nerve firing in response to distension was significantly attenuated (Cockayne, Dunn et al. 2005). In these experiments, as with my own studies examining multiunit afferent responses in $TRPV1^{-/-}$ (KO) mice, approximately 80% of distension induced firing persisted in the knockouts (Cockayne, Dunn et al. 2005). Based on these findings it is my opinion that the $P2X_{2/3}$ receptors on bladder afferent nerves contribute only a portion to the normal physiological response to distension and that this proportion of the response arises from urothelial afferents that are tension insensitive. Due of the similar level of afferent attenuation in both the $TRPV1^{-/-}$ and $P2X_{2/3}^{-/-}$ mouse it is tempting to speculate that the proportion of urothelial afferents contributing to the distension response is the same in both studies. Thus, I would propose that ATP is progressively released from the urothelium during distension with subsequent activation of purinergic receptors on urothelial afferents also expressing the TRPV1 receptor (Ferguson, Kennedy et al. 1997; Vlaskovska, Kasakov et al. 2001). Reduced ATP release from the urothelium in $TRPV1^{-/-}$ (KO) mice would also contribute to blunted afferent sensitivity in these animals.

The functional role of these distension insensitive but purinergic and capsaicin sensitive afferents is unclear. A large proportion of capsaicin sensitive afferents have been shown to colocalise with purinergic receptors and these are reported to respond both to high and low distension threshold (Rong, Spyer et al. 2002; Daly, Rong et al. 2007; Zagorodnyuk, Brookes et al. 2010). It is likely, despite contributing only a moderate proportion of the overall response to distension, that these afferents are essential to the normal micturition reflex. Due to their localisation within the bladder close to the urothelium, they are also in prime position to respond to factors released from the urothelium, including ATP. These endings may also be particularly important in bladder disorders when sensitisation of afferent pathways occurs. This view is supported by the observed upregulation of TRPV1 sensitive afferents seen in disease, and the corresponding symptomatic improvement following intravesical resiniferatoxin treatment (Apostolidis, Brady et al. 2005; Apostolidis, Popat et

al. 2005). As tachykinin neuropeptides are primarily contained within CSPANS, tachykinin immunoreactivity is significantly increased in the sub-urothelium of patients with idiopathic detrusor overactivity (Moore K H 1992; Smet, Moore et al. 1997), and tachykinins have been shown to enhance excitability in capsaicin-responsive DRG neurons (Sculptoreanu and de Groat 2007) but not alter the mechanosensitive response to distension, it is still possible that a proportion of the positive effects elicited by resiniferatoxin on bladder disorders is mediated by depletion of tachykinins from peripheral terminals.

My findings during the course of this PhD study are that in the normal bladder the majority of the afferent response is related to intravesical pressure/bladder wall tension, but there is also a proportion that is tension insensitive.

The role of the urothelium

The concept of bladder afferent nerves responding to ATP is well established and is considered to be mediated via release from the urothelium. Results obtained showing that baseline afferent firing increases following the intravesical instillation of αβMe-ATP is consistent with that view and prompted a series of investigations in which receptor expression and mediator release was directly investigated.

I was therefore able to show by RT-qPCR that the urothelium expresses purinergic receptors and furthermore in functional studies, provide evidence towards an indirect role for the urothelium in sensory signalling. These studies contribute to the growing view that the urothelium is not a simple barrier but plays a role as a sensory structure.

As was shown in chapters 4 and 5 of this thesis, a number of mediators including acetylcholine, ATP and PGE_2 are released from the urothelium and of these; the release of ATP is the most sensitive to bladder distension. Other studies have also shown nitric oxide release (Birder, Nakamura et al. 2002), but this was not explored in the current study. ATP is released from the urothelium during distension and has a number of proposed roles. Vesicular release of ATP during stretch is found to be essential to maintaining the barrier function of the urothelium as the bladder expands (Wang, Lee et al. 2005). This autofeedback mechanism allows membrane trafficking during stretch and relaxation (Wang, Lee et al. 2005). In addition, the presence of functional purinergic receptors within the underlying structures of the bladder including the detrusor smooth muscle, interstitial cells, and tension

insensitive afferent nerve fibers indicates that non-neuronal ATP has a major role to play in the bladder.

The level of distension-evoked ATP release increases significantly with age (Yoshida, Homma et al. 2001; Collins, Daly et al. 2013), a known risk factor for all lower urinary tract symptoms. ATP is also increased in a number of disease models including spinal cord injury, cyclophosphamide induced inflammation, and feline interstitial cystitis (Sun and Chai 2002; Khera, Somogyi et al. 2004; Smith, Vemulakonda et al. 2005; Smith, Gangitano et al. 2008). Instillation of a number of current therapeutic agents including DMSO, heparin, and Botox, into the bladder results in a significant reduction in distension-evoked ATP release and is associated with an improvement in lower urinary tract symptoms. ATP release is also altered in human luminal samples from patients with OAB and has been proposed as a biomarker for OAB detection (Cheng, Mansfield et al. 2013; Silva-Ramos, Silva et al. 2013).

Evidence supporting a significant role for the urothelium in mechanotransduction comes from a number of experiments performed in this thesis. Firstly, deletion of the TRPV1 receptor abolishes distension evoked ATP release from the urothelium and contributes to the sensory deficit observed during bladder distension in the $TRPV1^{-/-}$ (KO) mouse. TRPV1-mediated ATP release in urothelial cells has been reported by a number of authors (Birder, Kanai et al. 2001; Sadananda, Shang et al. 2009; Dunning-Davies, Fry et al. 2013) but as TRPV1 is not thought to directly activate mechanically gated channels, the mechanisms responsible for these actions are uncertain. Data from both calcium imaging experiments and biochemical release assays provide evidence for the underlying mechanisms of distension-induced ATP release which is consistent with previous data on the effect of pharmacological manipulation of vesicular release (Birder, Barrick et al. 2003; Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011; Collins, Daly et al. 2013). Moreover, Birder et al (2002) did not see an increase in membrane capacitance in $TRPV1^{-/-}$ (KO) mouse bladders upon distension suggesting that the actions of TRPV1 may be upstream of vesical fusion (Birder, Nakamura et al. 2002). These results are consistent with Wang et al (2005) who proposed that vesicular ATP release is essential to the maintenance of the urothelial barrier, and thus a loss of ATP release would render the barrier more permeable. Purinergic receptors have also been identified on the suburothelial interstitial cells where they make close contact with afferent endings (Wiseman, Fowler et al. 2003), and are able to depolarise afferents in the surrounding area up to several hundred microns away (Sui, Wu et al. 2004). With this evidence, it is possible that the activation of suburothelial cells is able to create a functional link between urothelial ATP and afferent neurons and has the potential to influence their excitability.

The specificity of ATP in mediating direct afferent nerve transduction was made apparent by studies utilizing intravesical infusion of NKA. Intravesical NKA had no effect on ATP release, either in the basal state, or during bladder distension, and was not able to influence spontaneous afferent excitability. However, NKA was able to significantly reduce ACh release by a mechanism that has yet to be determined. As afferent nerve activity was not directly affected this suggests that urothelial ACh is not a major stimulus for suburothelial afferent nerves and that ATP is the major player. Intravesical NKA was however, able to significantly alter the overall afferent response to distension through a novel pathway that could potentially involve suburothelial interstitial cells. In this hypothesis, the interstitial cells provide a functional link between the mediators released from the urothelium and the contractile properties of the detrusor smooth muscle. Interstitial cells have been shown to express both purinergic (Cheng, Scigalla et al. 2011; Li, Xue et al. 2013) and muscarinic receptors (Johnston, Carson et al. 2008). It is proposed that under normal conditions, the role of ACh acting on interstitial cells is to tonically inhibit contraction of the bladder as it fills. A decreased release of urothelial ACh could therefore allow the bladder to develop more frequent and higher amplitude spontaneous contractions that are coupled to bursts in afferent nerve activity. This is counterintuitive since muscarinic blockers are the mainstay of current therapy for OAB (Abrams and Andersson 2007). However, this action is likely to be mediated at the level of smooth muscle which expresses M2 and M3 receptors, without which parasympathetically mediated bladder contractions are lost (Chess-Williams, Chapple et al. 2001). In this respect, it has been shown that ACh significantly inhibited the afferent response to bladder distension while concurrently reducing bladder compliance in mice (Daly, Chess-Williams et al. 2010). Thus, it is hypothesised that tachykinins can stimulate phasic contractile activity by inhibiting ACh release, via a process of disinhibition. Under normal physiological conditions there is the potential for multiple mechanisms involving muscle contractility and urothelial release mechanisms acting on tension-sensitive and tension-insensitive afferents driving sensory afferent activity. The balance of these mechanisms may alter in a number of bladder diseases leading to increases in reflex bladder contractility and sensory signalling.

A potential role for interstitial cells in the control of detrusor smooth muscle and afferent nerve activity has been discussed on a number of occasions although this thesis has not examined their role directly. Nonetheless, there are a number of bladder conditions in which ATP release is thought to increase, accompanied by increases in reflex bladder activity (Smith, Vemulakonda et al. 2005; Smith, Gangitano et al. 2008) which are reported to involve interstitial cells. Human interstitial cell cultures have been shown to respond to nanomolar concentrations of ATP and P2Y receptors have been implicated in mediating spontaneous and electrical activity of guinea-pig interstitial cells to ATP

(Sui, Wu et al. 2006). ATP induces an intracellular Ca^{2+} transient (Wu, Sui et al. 2004; Cheng, Scigalla et al. 2011; Li, Xue et al. 2013) which propagates through the interstitial cell network (Fry, Sui et al. 2007), and similar to that of gastrointestinal tract, is hypothesised to coordinate myogenic contractions.

Non-neuronal ATP released from the bladder is likely to have an important role in the control of sensory afferent discharge. It is not likely to provide a direct mechanism controlling a large proportion of the afferent response but its ubiquitous expression and functional properties suggest that the coordination of ATP release from the urothelium and action on interstitial cells, afferent nerves and detrusor smooth muscle, is crucial to normal voiding reflexes and changes are a key marker in disease states.

As the importance of ATP release in sensory afferent firing becomes apparent, and the ability of the urothelium to autoregulate ATP release becomes established, the possibility of new drugs that target purinergic receptors becomes feasible. Moreover, the results showing that these receptors are present on the urothelium and that they modulate intracellular calcium concentration, an essential intracellular event linked to distension evoked ATP release (Matsumoto-Miyai, Kagase et al. 2009; Matsumoto-Miyai, Kagase et al. 2011; Dunning-Davies, Fry et al. 2013), suggests the urothelium itself could be a therapeutic target. One advantage of the urothelium as a target for pharmaceutical agents is that drugs acting from the luminal side of the bladder have limited access to systemic and CNS mechanisms and therefore carry a reduced risk of side effects, the most commonly cited reason for a discontinuation rate of 75% of patients after 12 months with antimuscarinics (Wagg, Compion et al. 2012; Veenboer and Bosch 2013)(Wagg 2012, Veenboer 2013).

Conclusion

As with any avenues of scientific research, the work in this thesis has generated a number of questions and interesting avenues for future investigation. The major stimulus for afferent signalling is bladder distension which results in an increase in transmural tension. However, my findings have shown that intravesical application of agonists acting at the level of the urothelium has the potential to alter this dynamic process. The development and use of isolated cell models has been key in determining the molecular mechanisms underlying urothelial stimulation and responses. However, by applying these principles to the whole bladder it has been possible to evaluate the impact of the cellular events for overall sensory signalling. In this regard, determining the physiology of the urothelium and its interactions with associated cells would be, in my opinion, the key to determining therapeutic targets for the treatment of overactive bladder.

One potential target for urothelially released ATP are the bladder afferent nerves which express $P2X_2$ and $P2X_3$ receptors. These are ligand-gated ion channels that are fast acting and undergo rapid desensitisation following agonist binding (Virginio, MacKenzie et al. 1999; North 2002). This was shown to be the case in experiments carried out in this thesis, whereby application of αβMe-ATP to thoraco-lumbar and lumbo-sacral DRG neurons induced marked increases in intracellular calcium on the first application, but subsequent responses were significantly attenuated, a result which has been observed before using whole cell patch clamp electrophysiology (Cockayne, Hamilton et al. 2000; Cockayne, Dunn et al. 2005). Considering that the mechanosensitive role of $P2X_3$ receptors on bladder afferent nerves is presumed to be through ATP released from the urothelium, and this release of ATP has been shown to be both continual and to increase in concentration during bladder stretch (Vlaskovska, Kasakov et al. 2001), it is likely that receptor desensitisation is a limiting factor in sensory signalling. Nevertheless a role for urothelially released ATP and its metabolites is most certainly important in mechanosensation, but the idea that $P2X_3$ receptors upon the afferent nerves are the mediators of significant mechanosensation is over simplified. As this thesis has demonstrated, other purinergic receptor mechanisms are likely involved, interacting with various cell types and other mediators to offer a dynamic afferent sensitivity.

The results of this thesis support the concept for a role whereby the urothelium releases a number of agents including ATP which play a coordinated role to influence detrusor muscle contraction, afferent nerve activity, and bladder compliance. The use of experimental techniques that provide information regarding afferent nerve activity are essential to determining the physiological roles of

the bladder, as this is the major stimulus for the initiation of the micturition reflex. Understanding how these mechanisms are influenced in disease is the next challenge.

BIBLIOGRAPHY

Abdel-Gawad, M., S. B. Dion, et al. (2001). "Evidence of a peripheral role of neurokinins in detrusor hyperreflexia: a further study of selective tachykinin antagonists in chronic spinal injured rats." The Journal of Urology **165**(5): 1739-1744.

Abdel-Karim, A. M., H. G. Barthlow, et al. (2005). "Effects of M274773, a neurokinin-2 receptor antagonist, on bladder function in chronically spinalized rats." The Journal of Urology **174**(4, Part 1): 1488-1492.

Abrams, P. and K.-E. Andersson (2007). "Muscarinic receptor antagonists for overactive bladder." BJU International **100**(5): 987-1006.

Abrams, P., L. Cardozo, et al. (2002). "The standardisation of terminology of lower urinary tract function: report from the standardisation sub-committee of the International Continence Society." Neurourology and Urodynamics **21**(2): 167-178.

Ahern, G. P. (2003). "Activation of TRPV1 by the satiety factor oleoylethanolamide." Journal of Biological Chemistry **278**(33): 30429-30434.

Aizawa, N., Y. Igawa, et al. (2011). "Effects of nitric oxide on the primary bladder afferent activities of the rat with and without intravesical acrolein treatment." European Urology **59**(2): 264-271.

Alexander, S. P. H., A. Mathie, et al. (2011). "Ion channels." British Journal of Pharmacology **164**: S137-S174.

Amaya, F., G. Shimosato, et al. (2004). "NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia." European Journal of Neuroscience **20**(9): 2303-2310.

Anderson, K. E. (1993). "Pharmacology of lower urinary tract smooth muscles and penile erectile tissues." Pharmacological Reviews **45**(3): 253-308.

Andersson, K.-E. (2002). "Bladder activation: afferent mechanisms." Urology **59**(5, Supplement 1): 43-50.

Andersson, K.-E. (2010). "Detrusor myocyte activity and afferent signaling." Neurourology and Urodynamics **29**(1): 97-106.

Andersson, K.-E. and A. Arner (2004). "Urinary bladder contraction and relaxation: physiology and pathophysiology." Physiological Reviews **84**(3): 935-986.

Andersson, K.-E. and A. J. Wein (2004). "Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence." Pharmacological Reviews **56**(4): 581-631.

Andersson, K. E. and M. Yoshida (2003). "Antimuscarinics and the overactive detrusor—which is the main mechanism of action?" European Urology **43**(1): 1-5.

Apodaca, G. (2004). "The uroepithelium: not just a passive barrier." Traffic **5**(3): 117-128.

Apostolidis, A., C. M. Brady, et al. (2005). "Capsaicin receptor TRPV1 in urothelium of neurogenic human bladders and effect of intravesical resiniferatoxin." Urology **65**(2): 400-405.

Apostolidis, A., G. E. Gonzales, et al. (2006). "Effect of intravesical resiniferatoxin (RTX) on lower urinary tract symptoms, urodynamic parameters, and quality of life of patients with urodynamic increased bladder sensation." European Urology **50**(6): 1299-1305.

Apostolidis, A., R. Popat, et al. (2005). "Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity." The Journal of Urology **174**(3): 977-983.

Avelino, A., C. Cruz, et al. (2002). "Vanilloid receptor 1 expression in the rat urinary tract." Neuroscience **109**(4): 787-798.

Avelino, A. and F. Cruz (2000). "Peptide immunoreactivity and ultrastructure of rat urinary bladder nerve fibers after topical desensitization by capsaicin or resiniferatoxin." Autonomic Neuroscience **86**(1–2): 37-46.

Avelino, A. and F. Cruz (2006). "TRPV1 (vanilloid receptor) in the urinary tract: expression, function and clinical applications." Naunyn-Schmiedeberg's Archives of Pharmacology **373**(4): 287-299.

Azadzoi, K. M., Z. M. Radisavljevic, et al. (2008). "Effects of ischemia on tachykinin-containing nerves and neurokinin receptors in the rabbit bladder." Urology **71**(5): 979-983.

Bahadory, F., K. H. Moore, et al. (2013). "Gene expression of muscarinic, tachykinin and purinergic receptors in porcine bladder: comparison with cultured cells." Frontiers in Pharmacology **4**.

Bayliss, M., C. Wu, et al. (1999). "A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders." The Journal of Urology **162**(5): 1833-1839.

Beckel, J. M. and L. A. Birder (2012). "Differential expression and function of nicotinic acetylcholine receptors in the urinary bladder epithelium of the rat." The Journal of Physiology **590**(6): 1465-1480.

Beckel, J. M., A. Kanai, et al. (2005). "Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells." American Journal of Physiology - Renal Physiology **290**(1): F103-F110.

Bhave, G., W. Zhu, et al. (2002). "cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation." Neuron **35**(4): 721-731.

Biers, S. M., J. M. Reynard, et al. (2006). "The functional effects of a c-kit tyrosine inhibitor on guinea-pig and human detrusor." BJU International **97**(3): 612-616.

Birder, L. and W. de Groat (1992). "Increased c-fos expression in spinal neurons after irritation of the lower urinary tract in the rat." The Journal of Neuroscience **12**(12): 4878-4889.

Birder, L., W. de Groat, et al. (2010). "Neural control of the lower urinary tract: peripheral and spinal mechanisms." Neurourology and Urodynamics **29**(1): 128-139.

Birder, L. A. (2006). "Urinary bladder urothelium: molecular sensors of chemical/thermal/mechanical stimuli." Vascular Pharmacology **45**(4): 221-226.

Birder, L. A. (2010). "Urothelial signaling." Autonomic Neuroscience **153**(1–2): 33-40.

Birder, L. A., S. R. Barrick, et al. (2003). "Feline interstitial cystitis results in mechanical hypersensitivity and altered ATP release from bladder urothelium." American Journal of Physiology - Renal Physiology **285**(3): F423-F429.

Birder, L. A., A. J. Kanai, et al. (2001). "Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells." Proceedings of the National Academy of Sciences **98**(23): 13396-13401.

Birder, L. A., Y. Nakamura, et al. (2002). "Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1." Nat Neuroscience **5**(9): 856-860.

Birder, L. A., H. Z. Ruan, et al. (2004). "Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis." American Journal of Physiology - Renal Physiology **287**(5): F1084-F1091.

Birder, L. A., A. Wolf-Johnston, et al. (2005). "Altered inducible nitric oxide synthase expression and nitric oxide production in the bladder of cats with feline interstitial cystitis." The Journal of Urology **173**(2): 625-629.

Blok, B. F., A. T. Willemsen, et al. (1997). "A PET study on brain control of micturition in humans." Brain **120**(1): 111-121.

Bonev, A. D. and M. T. Nelson (1993). "ATP-sensitive potassium channels in smooth muscle cells from guinea pig urinary bladder." American Journal of Physiology - Cell Physiology **264**(5): C1190-C1200.

Boudreault, F. and R. Grygorczyk (2002). "Cell swelling-induced ATP release and gadolinium-sensitive channels." American Journal of Physiology - Cell Physiology **282**(1): C219-C226.

Bradbury, E. J., G. Burnstock, et al. (1998). "The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor." Molecular and Cellular Neuroscience **12**(4–5): 256-268.

Brady, C. M., A. Apostolidis, et al. (2004). "P2X3-immunoreactive nerve fibres in neurogenic detrusor overactivity and the effect of intravesical resiniferatoxin." European Urology **46**(2): 247-253.

Brady, C. M., A. N. Apostolidis, et al. (2004). "Parallel changes in bladder suburothelial vanilloid receptor TRPV1 and pan-neuronal marker PGP9.5 immunoreactivity in patients with neurogenic detrusor overactivity after intravesical resiniferatoxin treatment." BJU International **93**(6): 770-776.

Brechenmacher, C., Y. Larmet, et al. (1998). "Cultured rat sensory neurones express functional tachykinin receptor subtypes 1, 2 and 3." Neuroscience Letters **241**(2–3): 159-162.

Brierley, S. M. (2010). "Molecular basis of mechanosensitivity." Autonomic Neuroscience **153**(1–2): 58-68.

Brierley, S. M., R. C. W. Jones Iii, et al. (2004). "Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice." Gastroenterology **127**(1): 166-178.

Buffington, C. A. T. and S. A. Wolfe Jr (1998). "High affinity binding sites for [(3) H]substance p in urinary bladders of cats with interstitial cystitis." The Journal of Urology **160**(2): 605-611.

Burcher, E, et al. (2000). Autoradiographic localization of tachykinin and calcitonin gene-related peptide receptors in adult urinary bladder. New York, NY, ETATS-UNIS, Elsevier.

Burcher, E., X.-P. Zeng, et al. (2000). "Autoradiographic localization of tachykinin and calcitonin gene-related peptide receptors in adult urinary bladder." The Journal of Urology **163**(1): 331-337.

Burnstock, G. (1999). "Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction." Journal of Anatomy **194**(3): 335-342.

Burnstock, G. (2000). "P2X receptors in sensory neurones." British Journal of Anaesthesia **84**(4): 476-488.

Burnstock, G. (2001). Purinergic Signalling in Lower Urinary Tract. Purinergic and Pyrimidinergic Signalling I. M. Abbracchio and M. Williams, Springer Berlin Heidelberg. **151 / 1:** 423-515.

Burnstock, G. (2009). "Purinergic cotransmission." Experimental Physiology **94**(1): 20-24.

Burnstock, G., G. Campbell, et al. (1970). "Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut." British Journal of Pharmacology **40**(4): 668-688.

Bushfield, M., M. Metcalfe, et al. (1995). "Activation of the micturition reflex by NK2 receptor stimulation in the anaesthetized guinea-pig." British Journal of Pharmacology **115**(6): 875-882.

Callsen-Cencic, P. and S. Mense (1997). "Expression of neuropeptides and nitric oxide synthase in neurones innervating the inflamed rat urinary bladder." Journal of the Autonomic Nervous System **65**(1): 33-44.

Candenas, L., A. Lecci, et al. (2005). "Tachykinins and tachykinin receptors: effects in the genitourinary tract." Life Sciences **76**(8): 835-862.

Caremel, R., S. Oger-Roussel, et al. (2010). "Nitric oxide/cyclic guanosine monophosphate signalling mediates an inhibitory action on sensory pathways of the micturition reflex in the rat." European Urology **58**(4): 616-625.

Carr, M., D. Hunter, et al. (2002). "Expression of tachykinins in nonnociceptive vagal afferent neurons during respiratory viral infection in guinea pigs." American Journal of Respiratory and Critical Care Medicine **165**(8): 1071-1075.

Caterina, M. J., A. Leffler, et al. (2000). "Impaired nociception and pain sensation in mice lacking the capsaicin receptor." Science **288**(5464): 306-313.

Cefalu, J. S., M. A. Guillon, et al. (2009). "Selective pharmacological blockade of the TRPV1 receptor suppresses sensory reflexes of the rodent bladder." The Journal of Urology **182**(2): 776-785.

Chancellor, M. B. and W. C. de Groat (1999). "Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder." The Journal of Urology **162**(1): 3-11.

Charrua, A., C. D. Cruz, et al. (2007). "Transient receptor potential vanilloid subfamily 1 is essential for the generation of noxious bladder input and bladder overactivity in cystitis." The Journal of Urology **177**(4): 1537-1541.

Charrua, A., C. D. Cruz, et al. (2009). "GRC-6211, a new oral specific TRPV1 antagonist, decreases bladder overactivity and noxious bladder input in cystitis animal models." The Journal of Urology **181**(1): 379-386.

Charrua, A., C. Reguenga, et al. (2009). "Functional transient receptor potential vanilloid 1 is expressed in human urothelial cells." The Journal of Urology **182**(6): 2944-2950.

Chen, C.-C., A. N. Akopian, et al. (1995). "A P2X purinoceptor expressed by a subset of sensory neurons." Nature **377**(6548): 428-431.

Cheng, C. L. and W. C. de Groat (2004). "The role of capsaicin-sensitive afferent fibers in the lower urinary tract dysfunction induced by chronic spinal cord injury in rats." Experimental Neurology **187**(2): 445-454.

Cheng, S., F. P. Scigalla, et al. (2011). "ATP enhances spontaneous calcium activity in cultured suburothelial myofibroblasts of the human bladder." PLoS ONE **6**(10): e25769.

Cheng, Y., K. Mansfield, et al. (2011). "Porcine bladder urothelial, myofibroblast and detrusor muscle cells: characterisation and ATP release." Frontiers in Pharmacology **2**.

Cheng, Y., K. J. Mansfield, et al. (2013). "Correlation between cystometric volumes, ATP release, and pH in women with overactive bladder versus controls." Neurourology and Urodynamics **32**(7): 969-973.

Chess-Williams, R. (2002). "Muscarinic receptors of the urinary bladder: detrusor, urothelial and prejunctional." Autonomic and Autacoid Pharmacology **22**(3): 133-145.

Chess-Williams, R., C. R. Chapple, et al. (2001). "The minor population of M3-receptors mediate contraction of human detrusor muscle in vitro." Journal of Autonomic Pharmacology **21**(5): 243-248.

Chopra, B., S. R. Barrick, et al. (2005). "Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat urinary bladder urothelium." The Journal of Physiology **562**(3): 859-871.

Chopra, B., J. Gever, et al. (2008). "Expression and function of rat urothelial P2Y receptors." American Journal of Physiology - Renal Physiology **294**(4): F821-F829.

Christensen, A. P. and D. P. Corey (2007). "TRP channels in mechanosensation: direct or indirect activation?" Nat Rev Neurosci **8**(7): 510-521.

Chu, H. W., M. Kraft, et al. (2000). "Substance P and its receptor neurokinin 1 expression in asthmatic airways." Journal of Allergy and Clinical Immunology **106**(4): 713-722.

Chuang, H.-h., E. D. Prescott, et al. (2001). "Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition." Nature **411**(6840): 957-962.

Chung, K., R. J. Schwen, et al. (1985). "Ureteral axon damage following subcutaneous administration of capsaicin in adult rats." Neuroscience Letters **53**(2): 221-226.

Clapham, D. E. (2003). "TRP channels as cellular sensors." Nature **426**(6966): 517-524.

Cockayne, D. A., P. M. Dunn, et al. (2005). "P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP." The Journal of Physiology **567**(2): 621-639.

Cockayne, D. A., S. G. Hamilton, et al. (2000). "Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice." Nature **407**(6807): 1011-1015.

Collins, V. M., D. M. Daly, et al. (2013). "OnabotulinumtoxinA significantly attenuates bladder afferent nerve firing and inhibits ATP release from the urothelium." BJU International **112**(7): 1018-1026.

Dalsgaard, C. J., A. Haegerstrand, et al. (1985). "Neurokinin A-like immunoreactivity in rat primary sensory neurons; coexistence with substance P." Histochemistry **83**(1): 37-39.

Daly, D., W. Rong, et al. (2007). "Bladder afferent sensitivity in wild-type and TRPV1 knockout mice." The Journal of Physiology **583**(2): 663-674.

Daly, D. M., R. Chess-Williams, et al. (2010). "The inhibitory role of acetylcholine and muscarinic receptors in bladder afferent activity." European Urology **58**(1): 22-28.

Dang, K., K. Bielefeldt, et al. (2005). "Differential responses of bladder lumbosacral and thoracolumbar dorsal root ganglion neurons to purinergic agonists, protons, and capsaicin." The Journal of Neuroscience **25**(15): 3973-3984.

Davidson, R. A. and K. D. McCloskey (2005). "Morphology and localization of interstitial cells in the guinea pig bladder: Structural relationships with smooth muscle and neurons." The Journal of Urology **173**(4): 1385-1390.

Davis, J. B., J. Gray, et al. (2000). "Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia." Nature **405**(6783): 183-187.

de Groat, M. O. F., M. Yoshiyama, S. Smerin, C. Tai, M. B. Chancellor, N. Yoshimura, J. R. Roppolo, W. (2001). "Neural control of the urethra." Scandinavian Journal of Urology and Nephrology **35**(207): 35-43.

de Groat, W. C. (1997). "A neurologic basis for the overactive bladder." Urology **50**(6, Supplement 1): 36-52.

de Groat, W. C. (2006). "Integrative control of the lower urinary tract: preclinical perspective." British Journal of Pharmacology **147**(S2): S25-S40.

de Groat, W. C. and P. M. Lalley (1972). "Reflex firing in the lumbar sympathetic outflow to activation of vesical afferent fibres." The Journal of Physiology **226**(2): 289-309.

de Groat, W. C. and R. J. Theobald (1976). "Reflex activation of sympathetic pathways to vesical smooth muscle and parasympathetic ganglia by electrical stimulation of vesical afferents." The Journal of Physiology **259**(1): 223-237.

De Ridder, D., V. Chandiramani, et al. (1997). "Intravesical capsaicin as a treatment for refractory detrusor hyperreflexia: a dual center study with long-term followup." The Journal of Urology **158**(6): 2087-2092.

Dinis, P., A. Charrua, et al. (2004). "Intravesical resiniferatoxin decreases spinal c-fos expression and increases bladder volume to reflex micturition in rats with chronic inflamed urinary bladders." BJU International **94**(1): 153-157.

Dinis, P., A. Charrua, et al. (2004). "Anandamide-evoked activation of vanilloid receptor 1 contributes to the development of bladder hyperreflexia and nociceptive transmission to spinal dorsal horn neurons in cystitis." The Journal of Neuroscience **24**(50): 11253-11263.

Downie, J. W. and J. A. Armour (1992). "Mechanoreceptor afferent activity compared with receptor field dimensions and pressure changes in feline urinary bladder." Canadian Journal of Physiology and Pharmacology **70**(11): 1457-1467.

Drake, M., I. Harvey, et al. (2003). "Autonomous activity in the isolated guinea pig bladder." Experimental Physiology **88**(1): 19-30.

Drake, M. J., C. J. Fowler, et al. (2010). "Neural control of the lower urinary and gastrointestinal tracts: supraspinal CNS mechanisms." Neurourology and Urodynamics **29**(1): 119-127.

Drake, M. J., I. J. Harvey, et al. (2005). "Localized contractions in the normal human bladder and in urinary urgency." BJU International **95**(7): 1002-1005.

Dunn, P. M., Y. Zhong, et al. (2001). "P2X receptors in peripheral neurons." Progress in Neurobiology **65**(2): 107-134.

Dunning-Davies, B. M., C. H. Fry, et al. (2013). "The regulation of ATP release from the urothelium by adenosine and transepithelial potential." BJU International **111**(3): 505-513.

Everaerts, W., M. R. Sepúlveda, et al. (2009). "Where is TRPV1 expressed in the bladder, do we see the real channel?" Naunyn-Schmiedeberg's Archives of Pharmacology **379**(4): 421-425.

Everaerts, W., J. Vriens, et al. (2010). "Functional characterization of transient receptor potential channels in mouse urothelial cells." American Journal of Physiology - Renal Physiology **298**(3): F692-F701.

Ferguson, D. R., I. Kennedy, et al. (1997). "ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes-a possible sensory mechanism?" The Journal of Physiology **505**(Pt 2): 503-511.

Fields, R. D. and G. Burnstock (2006). "Purinergic signalling in neuron-glia interactions." Nat Rev Neurosci **7**(6): 423-436.

Ford, A. P. D. W., J. R. Gever, et al. (2006). "Purinoceptors as therapeutic targets for lower urinary tract dysfunction." British Journal of Pharmacology **147**(S2): S132-S143.

Fowler, C. J., D. Griffiths, et al. (2008). "The neural control of micturition." Nat Rev Neurosci **9**(6): 453-466.

Fry, C. H., E. Meng, et al. (2010). "The physiological function of lower urinary tract smooth muscle." Autonomic Neuroscience **154**(1–2): 3-13.

Fry, C. H., D. Skennerton, et al. (2002). "The cellular basis of contraction in human detrusor smooth muscle from patients with stable and unstable bladders." Urology **59**(5, Supplement 1): 3-12.

Fry, C. H., G. P. Sui, et al. (2007). "The function of suburothelial myofibroblasts in the bladder." Neurourology and Urodynamics **26**(S6): 914-919.

Fry, C. H., J. S. Young, et al. (2012). "Modulation of spontaneous activity in the overactive bladder: the role of P2Y agonists." American Journal of Physiology - Renal Physiology **302**(11): F1447-F1454.

Gabella, G. and C. Davis (1998). "Distribution of afferent axons in the bladder of rats." Journal of Neurocytology **27**(3): 141-155.

Gevaert, T., J. Vandepitte, et al. (2007). "TRPV1 is involved in stretch-evoked contractile changes in the rat autonomous bladder model: a study with piperine, a new TRPV1 agonist." Neurourology and Urodynamics **26**(3): 440-450.

Gillespie, J. I. (2004). "The autonomous bladder: a view of the origin of bladder overactivity and sensory urge." BJU International **93**(4): 478-483.

Gillespie, P. G. and R. G. Walker (2001). "Molecular basis of mechanosensory transduction." Nature **413**(6852): 194-202.

Giniatullin, R., A. Nistri, et al. (2008). "Molecular mechanisms of sensitization of pain-transducing P2X3 receptors by the migraine mediators CGRP and NGF." Molecular Neurobiology **37**(1): 83-90.

Girard, B., J. Tompkins, et al. (2012). "Effects of CYP-induced cystitis on PACAP/VIP and receptor expression in micturition pathways and bladder function in mice with overexpression of NGF in urothelium." Journal of Molecular Neuroscience **48**(3): 730-743.

Giuliani, S., R. Patacchini, et al. (1993). "Characterization of the tachykinin neurokinin-2 receptor in the human urinary bladder by means of selective receptor antagonists and peptidase inhibitors." Journal of Pharmacology and Experimental Therapeutics **267**(2): 590-595.

Gomez-Pinilla, P., M. Pozo, et al. (2011). "Aging differentially modifies agonist-evoked mouse detrusor contraction and calcium signals." AGE **33**(1): 81-88.

Goodman, M. B., E. A. Lumpkin, et al. (2004). "Molecules and mechanisms of mechanotransduction." The Journal of Neuroscience **24**(42): 9220-9222.

Guard, S., D. Pain, et al. (1993). "[125I]neurokinin A labels pharmacologically distinct populations of NK2 binding sites in hamster and rabbit urinary bladder." European Journal of Pharmacology **236**(3): 496.

Guo, A., D. A. Simone, et al. (2001). "Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons." European Journal of Neuroscience **14**(2): 293-304.

Guo, A., L. Vulchanova, et al. (1999). "Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites." European Journal of Neuroscience **11**(3): 946-958.

Habler, H. J., W. Janig, et al. (1993). "Receptive properties of myelinated primary afferents innervating the inflamed urinary bladder of the cat." Journal of Neurophysiology **69**(2): 395-405.

Häbler, H. J., W. Jänig, et al. (1990). "Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat." The Journal of Physiology **425**(1): 545-562.

Häbler, H. J., W. Jänig, et al. (1993). "Myelinated primary afferents of the sacral spinal cord responding to slow filling and distension of the cat urinary bladder." The Journal of Physiology **463**(1): 449-460.

Hall, J. M., J. M. Flowers, et al. (1992). "A pharmacological study of NK1 and NK2 tachykinin receptor characteristics in the rat isolated urinary bladder." British Journal of Pharmacology **107**(3): 777-784.

Hamill, O. P. and B. Martinac (2001). "Molecular basis of mechanotransduction in living cells." Physiological Reviews **81**(2): 685-740.

Hanna-Mitchell, A. T., J. M. Beckel, et al. (2007). "Non-neuronal acetylcholine and urinary bladder urothelium." Life Sciences **80**(24–25): 2298-2302.

Hanna-Mitchell, A. T., A. S. Wolf-Johnston, et al. (2013). "Effect of botulinum toxin A on urothelial-release of ATP and expression of SNARE targets within the urothelium." Neurourology and Urodynamics: n/a-n/a.

Harrington, A. M., P. A. Hughes, et al. (2011). "A novel role for TRPM8 in visceral afferent function." Pain **152**(7): 1459-1468.

Hashitani, H. and A. F. Brading (2003). "Ionic basis for the regulation of spontaneous excitation in detrusor smooth muscle cells of the guinea-pig urinary bladder." British Journal of Pharmacology **140**(1): 159-169.

Hashitani, H., A. F. Brading, et al. (2004). "Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder." British Journal of Pharmacology **141**(1): 183-193.

Hellwig, N., N. Albrecht, et al. (2005). "Homo- and heteromeric assembly of TRPV channel subunits." Journal of Cell Science **118**(5): 917-928.

Hökfelt, T., B. Pernow, et al. (2001). "Substance P: a pioneer amongst neuropeptides." Journal of Internal Medicine **249**(1): 27-40.

Holton, P. (1959). "The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves." The Journal of Physiology **145**(3): 494-504.

Homolya, L., T. H. Steinberg, et al. (2000). "Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia." The Journal of Cell Biology **150**(6): 1349-1360.

Hunskaar, S., K. Burgio, et al. (2003). "Epidemiology and natural history of urinary incontinence in women." Urology **62**(4, Supplement 1): 16-23.

Hunter, D., A. Myers, et al. (2000). "Nerve growth factor–induced phenotypic switch in guinea pig airway sensory neurons." American Journal of Respiratory and Critical Care Medicine **161**(6): 1985-1990.

Hwang, S. J., J. Min Oh, et al. (2005). "Expression of the vanilloid receptor TRPV1 in rat dorsal root ganglion neurons supports different roles of the receptor in visceral and cutaneous afferents." Brain Research **1047**(2): 261-266.

Ichikawa, H. and T. Sugimoto (2001). "VR1-immunoreactive primary sensory neurons in the rat trigeminal ganglion." Brain Research **890**(1): 184-188.

Igawa, Y., Y. Yamazaki, et al. (1999). "Functional and molecular biological evidence for a possible β3-adrenoceptor in the human detrusor muscle." British Journal of Pharmacology **126**(3): 819-825.

Igawa, Y., X. Zhang, et al. (2004). "Cystometric findings in mice lacking muscarinic M2 or M3 receptors." The Journal of Urology **172**(6, Part 1): 2460-2464.

Ikeda, Y., C. Fry, et al. (2007). "Role of gap junctions in spontaneous activity of the rat bladder." American Journal of Physiology - Renal Physiology **293**(4): F1018-F1025.

Ikeda, Y. and A. Kanai (2008). "Urotheliogenic modulation of intrinsic activity in spinal cord-transected rat bladders: role of mucosal muscarinic receptors." American Journal of Physiology - Renal Physiology **295**(2): F454-F461.

Ishizuka, O., Y. Igawa, et al. (1994). "Role of intrathecal tachykinins for micturition in unanaesthetized rats with and without bladder outlet obstruction." British Journal of Pharmacology **113**(1): 111-116.

Ishizuka, O., A. Mattiasson, et al. (1995). "Tachykinin effects on bladder activity in conscious normal rats." The Journal of Urology **154**(1): 257-261.

Jänig, W. and M. Koltzenburg (1990). "On the function of spinal primary afferent fibres supplying colon and urinary bladder." Journal of the Autonomic Nervous System **30, Supplement**(0): S89-S96.

Johnston, L., C. Carson, et al. (2008). "Cholinergic-induced Ca2+ signaling in interstitial cells of Cajal from the guinea pig bladder." American Journal of Physiology - Renal Physiology **294**(3): F645-F655.

Jones, C. A. and L. Nyberg (1997). "Epidemiology of interstitial cystitis." Urology **49**(5, Supplement 1): 2-9.

Jones, R. C. W., L. Xu, et al. (2005). "The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3." The Journal of Neuroscience **25**(47): 10981-10989.

Jordt, S.-E., M. Tominaga, et al. (2000). "Acid potentiation of the capsaicin receptor determined by a key extracellular site." Proceedings of the National Academy of Sciences **97**(14): 8134-8139.

Jung, J., S.-Y. Lee, et al. (2002). "Agonist recognition sites in the cytosolic tails of vanilloid receptor 1." Journal of Biological Chemistry **277**(46): 44448-44454.

Kajioka, S., S. Nakayama, et al. (2002). "Ca2+ channel properties in smooth muscle cells of the urinary bladder from pig and human." European Journal of Pharmacology **443**(1–3): 19-29.

Keast, J. R. and W. C. de Groat (1992). "Segmental distribution and peptide content of primary afferent neurons innervating the urogenital organs and colon of male rats." The Journal of Comparative Neurology **319**(4): 615-623.

Keast, J. R., M. Kawatani, et al. (1990). "Sympathetic modulation of cholinergic transmission in cat vesical ganglia is mediated by alpha 1- and alpha 2-adrenoceptors." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **258**(1): R44-R50.

Khera, M., G. T. Somogyi, et al. (2004). "Botulinum toxin A inhibits ATP release from bladder urothelium after chronic spinal cord injury." Neurochemistry International **45**(7): 987-993.

Kibble, A. M., J.F.B (1996). "Changes in mechanosensitivity and sensitization of urinary bladder afferents in the anaesthetized rat following administration of the selective NK-2 antagonist SR48968." The Journal of Physiology **497**(Suppl): 13P-19P.

Kibble, A. M., J.F.B (1996). "In vivo pelvic afferent responses to a neurokinin A agonist in the anaesthetized rat." The Journal of Physiology **491**(Suppl): 133P-151P.

King, B., C.-C. Chen, et al. (1997). "A role for calcineurin in the desensitization of the P2X3 receptor." NeuroReport **8**(5): 1099-1102.

Kiss, S., M. Yoshiyama, et al. (2001). "Impaired response to chemical irritation of the urinary tract in mice with disruption of the preprotachykinin gene." Neuroscience Letters **313**(1–2): 57-60.

Knight, G. E., P. Bodin, et al. (2002). "ATP is released from guinea pig ureter epithelium on distension." American Journal of Physiology - Renal Physiology **282**(2): F281-F288.

Koplas, P. A., R. L. Rosenberg, et al. (1997). "The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons." The Journal of Neuroscience **17**(10): 3525-3537.

Kruse, R., S. Säve, et al. (2012). "Adenosine triphosphate induced P2Y2 receptor activation induces proinflammatory cytokine release in uroepithelial cells." The Journal of Urology **188**(6): 2419-2425.

Kullmann, F. A., D. Artim, et al. (2008). "Heterogeneity of muscarinic receptor-mediated Ca2+ responses in cultured urothelial cells from rat." American Journal of Physiology - Renal Physiology **294**(4): F971-F981.

Kullmann, F. A., M. A. Shah, et al. (2009). "Functional TRP and ASIC-like channels in cultured urothelial cells from the rat." American Journal of Physiology - Renal Physiology **296**(4): F892-F901.

Kumar, V., C. R. Chapple, et al. (2010). "In vitro release of adenosine triphosphate from the urothelium of human bladders with detrusor overactivity, both neurogenic and idiopathic." European Urology **57**(6): 1087-1092.

Kumar, V., C. R. Chapple, et al. (2007). "Enhanced adenosine triphosphate release from the urothelium of patients with painful bladder syndrome: a possible pathophysiological explanation

" The Journal of Urology **178**(4): 1533-1536.

Lasanen, L. T., T. L. J. Tammela, et al. (1992). "The effect of acute distension on vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and substance P (SP) immunoreactive nerves in the female rat urinary bladder." Urological Research **20**(4): 259-263.

Lazarowski, E. R. and T. K. Harden (1999). "Quantitation of extracellular UTP using a sensitive enzymatic assay." British Journal of Pharmacology **127**(5): 1272-1278.

Lazarowski, E. R., L. Homolya, et al. (1997). "Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation." Journal of Biological Chemistry **272**(39): 24348-24354.

Lazzeri, M., M. Spinelli, et al. (1998). "Intravesical resiniferatoxin for the treatment of detrusor hyperreflexia refractory to capsaicin in patients with chronic spinal cord diseases." Scandinavian Journal of Urology and Nephrology **32**(5): 331-334.

Lazzeri, M., M. G. Vannucchi, et al. (2004). "Immunohistochemical evidence of vanilloid receptor 1 in normal human urinary bladder." European Urology **46**(6): 792-798.

Lecci, A., S. Giuliani, et al. (1993). "Evidence for a role of tachykinins as sensory transmitters in the activation of micturition reflex." Neuroscience **54**(3): 827-837.

Lecci, A., S. Giuliani, et al. (1994). "Involvement of spinal tachykinin NK1 and NK2 receptors in detrusor hyperreflexia during chemical cystitis in anaesthetized rats." European Journal of Pharmacology **259**(2): 129-135.

Lecci, A., S. Giuliani, et al. (1997). "MEN 11,420, a peptide tachykinin NK2 receptor antagonist, reduces motor responses induced by the intravesical administration of capsaicin in vivo." Naunyn-Schmiedeberg's Archives of Pharmacology **356**(2): 182-188.

Lecci, A., S. Giuliani, et al. (1998). "Bladder distension and activation of the efferent function of sensory fibres: similarities with the effect of capsaicin." British Journal of Pharmacology **124**(2): 259-266.

Lecci, A. and C. A. Maggi (2001). "Tachykinins as modulators of the micturition reflex in the central and peripheral nervous system." Regulatory Peptides **101**(1–3): 1-18.

Lecci, A., M. Tramontana, et al. (1998). "Effect of tachykinin NK2 receptor blockade on detrusor hyperreflexia induced by bacterial toxin in rats." The Journal of Urology **160**(1): 206-209.

Lee, H. Y., M. Bardini, et al. (2000). "Distribution of P2X receptors in the urinary bladder and the ureter of the rat." The Journal of Urology **163**(6): 2002-2007.

Lewis, S. A. (2000). "Everything you wanted to know about the bladder epithelium but were afraid to ask." American Journal of Physiology - Renal Physiology **278**(6): F867-F874.

Lewis, S. A., J. R. Berg, et al. (1995). "Modulation of epithelial permeability by extracellular macromolecules." Physiological Reviews **75**(3): 561-589.

Li, Y., L. Xue, et al. (2013). "Expression and electrophysiological characteristics of P2X3 receptors in interstitial cells of Cajal in rats with partial bladder outlet obstruction." BJU International **111**(5): 843-851.

Liu, B., F. Yang, et al. (2014). "Increased severity of inflammation correlates with elevated expression of TRPV1 nerve fibers and nerve growth factor on interstitial cystitis/bladder pain syndrome." Urologia Internationalis **92**(2): 202-208.

Liu, B., C. Zhang, et al. (2005). "Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate." The Journal of Neuroscience **25**(19): 4835-4843.

Liu, F., N. Takahashi, et al. (2009). "Expression of P2X3 purinoceptors in suburothelial myofibroblasts of the normal human urinary bladder." International Journal of Urology **16**(6): 570-575.

Liu, M., Y.-F. Xu, et al. (2013). "Epigallocatechin gallate attenuates interstitial cystitis in human bladder urothelium cells by modulating purinergic receptors." Journal of Surgical Research **183**(1): 397-404.

Lomax, A. E. and J. B. Furness (2000). "Neurochemical classification of enteric neurons in the guinea-pig distal colon." Cell and Tissue Research **302**(1): 59-72.

Longmore, J. and R. G. Hill (1992). "Characterisation of NK receptors in guinea-pig urinary bladder smooth muscle: use of selective antagonists." European Journal of Pharmacology **222**(1): 167-170.

Luber, K. M. (2004). "The definition, prevalence, and risk factors for stress urinary incontinence." Reviews in urology **6 Suppl 3**: S3-9.

Maggi, C. A. (1993). "The dual, sensory and “efferent” function of the capsaicin-sensitive primary sensory neurons in the urinary bladder and urethra." The autonomic nervous system **3**: 383-422.

Maggi, C. A. (1995). "The mammalian tachykinin receptors." General Pharmacology: The Vascular System **26**(5): 911-944.

Maggi, C. A. (1997). "The effects of tachykinins on inflammatory and immune cells." Regulatory Peptides **70**(2–3): 75-90.

Maggi, C. A., S. Giuliani, et al. (1989). "Multiple sources of calcium for contraction of the human urinary bladder muscle." British Journal of Pharmacology **98**(3): 1021-1031.

Maggi, C. A., S. Giuliani, et al. (1991). "Facilitation of reflex micturition by intravesical administration of [beta Ala8]-neurokinin A (4-10), a selective NK-2 tachykinin receptor agonist." The Journal of Urology **145**(1): 184-187.

Maggi, C. A., R. Patacchini, et al. (1991). "Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission." British Journal of Pharmacology **103**(2): 1535-1541.

Maggi, C. A. and T. W. Schwartz (1997). "The dual nature of the tachykinin NK1 receptor." Trends in Pharmacological Sciences **18**(10): 351-354.

Malykhina, A., Q. Lei, et al. (2012). "VEGF induces sensory and motor peripheral plasticity, alters bladder function, and promotes visceral sensitivity." BMC Physiology **12**(1): 15.

Mansfield, K. J., L. Liu, et al. (2005). "Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT–PCR: changes in ageing." British Journal of Pharmacology **144**(8): 1089-1099.

Mar, L., F.-C. Yang, et al. (2012). "Genetic marking and characterization of Tac2-expressing neurons in the central and peripheral nervous system." Molecular Brain **5**(1): 3.

Martin, T. V., M. A. Wheeler, et al. (1997). "Neurokinin induced inositol phosphate production in guinea pig bladder." The Journal of Urology **157**(3): 1098-1102.

Martini, F. (2001). Fundamentals of anatomy and physiology. New Jersey, Prentice Hall.

Maserejian, N., V. Minassian, et al. (2014). "Treatment status and risk factors for incidence and persistence of urinary incontinence in women." International Urogynecology Journal: 1-8.

Matsui, M., D. Motomura, et al. (2002). "Mice lacking M2 and M3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable." The Journal of Neuroscience **22**(24): 10627-10632.

Matsumoto-Miyai, K., A. Kagase, et al. (2009). "Extracellular Ca2+ regulates the stimulus-elicited ATP release from urothelium." Autonomic Neuroscience **150**(1–2): 94-99.

Matsumoto-Miyai, K., A. Kagase, et al. (2011). "Store-operated Ca2+ entry suppresses distention-induced ATP release from the urothelium." American Journal of Physiology - Renal Physiology **300**(3): F716-F720.

Matta, J. A., R. L. Miyares, et al. (2007). "TRPV1 is a novel target for omega-3 polyunsaturated fatty acids." The Journal of Physiology **578**(2): 397-411.

McCloskey, K. D. (2010). "Interstitial cells in the urinary bladder—localization and function." Neurourology and Urodynamics **29**(1): 82-87.

McCloskey, K. D., U. A. Anderson, et al. (2009). "Comparison of mechanical and electrical activity and interstitial cells of Cajal in urinary bladders from wild-type and W/Wv mice." British Journal of Pharmacology **156**(2): 273-283.

Mckinley, M. a. V. O. L. (2009). Human anatomy. New York, McGraw Hill.

Meini, S. and C. A. Maggi (1994). "Evidence for a capsaicin-sensitive, tachykinin-mediated, component in the NANC contraction of the rat urinary bladder to nerve stimulation." British Journal of Pharmacology **112**(4): 1123-1131.

Mersdorf, A., R. A. Schmidt, et al. (1992). "Intrathecal administration of substance P in the rat: the effect on bladder and urethral sphincteric activity." Urology **40**(1): 87-96.

Milsom, I., P. Abrams, et al. (2001). "How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study." BJU International **87**(9): 760-766.

Minke, B. and B. Cook (2002). "TRP channel proteins and signal transduction." Physiological Reviews **82**(2): 429-472.

Minsky, B. and F. Chlapowski (1978). "Morphometric analysis of the translocation of lumenal membrane between cytoplasm and cell surface of transitional epithelial cells during the expansion-contraction cycles of mammalian urinary bladder." The Journal of Cell Biology **77**(3): 685-697.

Mo, G., L.-P. Bernier, et al. (2009). "Subtype-specific regulation of P2X3 and P2X2/3 receptors by phosphoinositides in peripheral nociceptors." Molecular Pain **5**(1): 47.

Mo, G., J. C. Peleshok, et al. (2013). "Control of P2X3 channel function by metabotropic P2Y2 UTP receptors in primary sensory neurons." Molecular Pharmacology **83**(3): 640-647.

Mohapatra, D. P. and C. Nau (2005). "Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase." Journal of Biological Chemistry **280**(14): 13424-13432.

Montgomery, B. and C. Fry (1992). "The action potential and net membrane currents in isolated human detrusor smooth muscle cells." The Journal of Urology **147**(1): 176.

Moore K H , G. S. A., Dixon J S, Richmond D H, Sutherst J R (1992). "Increase in presumptive sensory nerves of the urinary bladder in idiopathic detrusor instability." Britixh journal of urology **70**(4): 370-372.

Morrison, J. (1999). "The activation of bladder wall afferent nerves." Experimental Physiology **84**(1): 131-136.

Mukerji, G., Y. Yiangou, et al. (2006). "Transient receptor potential vanilloid receptor subtype 1 in painful bladder syndrome and its correlation with pain." The Journal of Urology **176**(2): 797-801.

Mussap, C. J., D. P. Geraghty, et al. (1993). "Tachykinin receptors: a radioligand binding perspective." Journal of Neurochemistry **60**(6): 1987-2009.

Nagy, I., P. Sántha, et al. (2004). "The role of the vanilloid (capsaicin) receptor (TRPV1) in physiology and pathology." European Journal of Pharmacology **500**(1–3): 351-369.

Namasivayam, Eardley, et al. (1999). "Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat." BJU International **84**(7): 854-860.
Neumann, S., T. P. Doubell, et al. (1996). "Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons." Nature **384**(6607): 360-364.
North, R. A. (2002). "Molecular physiology of P2X receptors." Physiological Reviews **82**(4): 1013-1067.
O'Reilly, B. A., A. H. Kosaka, et al. (2002). "P2X receptors and their role in female idiopathic detrusor instability." The Journal of Urology **167**(1): 157-164.
Obreja, O., M. Schmelz, et al. (2002). "Interleukin-6 in combination with its soluble IL-6 receptor sensitises rat skin nociceptors to heat, in vivo." Pain **96**(1–2): 57-62.
Ochodnický, P., M. B. Michel, et al. (2013). "Bradykinin modulates spontaneous nerve growth factor production and stretch-induced ATP release in human urothelium." Pharmacological Research **70**(1): 147-154.
Olsen, S., J. Stover, et al. (2011). "Examining the role of mechanosensitive ion channels in pressure mechanotransduction in rat bladder urothelial cells." Annals of Biomedical Engineering **39**(2): 688-697.
Oprée, A. and M. Kress (2000). "Involvement of the proinflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related reptide release from rat skin." The Journal of Neuroscience **20**(16): 6289-6293.
Ost, D., T. Roskams, et al. (2002). "Topography of the vanilloid receptor in the human bladder: more than just the nerve fibers." The Journal of Urology **168**(1): 293-297.
Ouslander, J. G. (2004). "Management of overactive bladder." New England Journal of Medicine **350**(8): 786-799.
Pandita, R. K. and K.-E. Andersson (2002). "Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats." The Journal of Urology **168**(3): 1230-1234.
Pang, X., J. Marchand, et al. (1995). "Increased number of substance P positive nerve fibres in interstitial cystitis." British Journal of Urology **75**(6): 744-750.
Paré, M., R. Elde, et al. (2001). "The Meissner corpuscle revised: a multiafferented mechanoreceptor with nociceptor immunochemical properties." The Journal of Neuroscience **21**(18): 7236-7246.
Parsons, C. L. (2007). "The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis." Urology **69**(4, Supplement): S9-S16.
Parsons, C. L., R. A. Greene, et al. (2005). "Abnormal urinary potassium metabolism in patients with interstitial cystitis." The Journal of Urology **173**(4): 1182-1185.
Pennefather, J. N., A. Lecci, et al. (2004). "Tachykinins and tachykinin receptors: a growing family." Life Sciences **74**(12): 1445-1463.
Philyppov, I. B., O. N. Paduraru, et al. (2012). "Modulation of TRPV1-dependent contractility of normal and diabetic bladder smooth muscle by analgesic toxins from sea anemone Heteractis crispa." Life Sciences **91**(19–20): 912-920.
Pinto, R., T. Lopes, et al. (2010). "Trigonal injection of botulinum toxin A in patients with refractory bladder pain syndrome/interstitial cystitis." European Urology **58**(3): 360-365.
Piper, A. S. and R. J. Docherty (2000). "One-way cross-desensitization between P2X purinoceptors and vanilloid receptors in adult rat dorsal root ganglion neurones." The Journal of Physiology **523**(3): 685-696.
Quinn, T., C. Collins, et al. (2004). "Mechanisms of neurokinin A- and substance P-induced contractions in rat detrusor smooth muscle in vitro." BJU International **94**(4): 651-657.
Regoli, D., A. Boudon, et al. (1994). "Receptors and antagonists for substance P and related peptides." Pharmacological Reviews **46**(4): 551-599.
Rizzo, C. A. and J. A. Hey (2000). "Activity of nonpeptide tachykinin antagonists on neurokinin a induced contractions in dog urinary bladder." The Journal of Urology **163**(6): 1971-1974.
Roberts, M. M. (2008). "Neurophysiology in neurourology." Muscle & Nerve **38**(1): 815-836.

Rong, W., K. Hillsley, et al. (2004). "Jejunal afferent nerve sensitivity in wild-type and TRPV1 knockout mice." The Journal of Physiology **560**(3): 867-881.
Rong, W., K. M. Spyer, et al. (2002). "Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder." The Journal of Physiology **541**(2): 591-600.
Roosen, A., S. N. Datta, et al. (2009). "Suburothelial myofibroblasts in the human overactive bladder and the effect of botulinum neurotoxin type A treatment." European Urology **55**(6): 1440-1449.
Rortveit, G., A. K. Daltveit, et al. (2003). "Urinary incontinence after vaginal delivery or cesarean section." New England Journal of Medicine **348**(10): 900-907.
Rosenbaum, T., A. Gordon-Shaag, et al. (2004). "Ca2+/calmodulin modulates TRPV1 activation by capsaicin." The Journal of General Physiology **123**(1): 53-62.
Ruggieri, M. R., S. Filer-Maerten, et al. (2000). "Role of neurokinin receptors in the behavioral effect of intravesical antigen infusion in guinea pig bladder." The Journal of Urology **164**(1): 197-202.
Saban, M., C. Davis, et al. (2011). "VEGF signaling mediates bladder neuroplasticity and inflammation in response to BCG." BMC Physiology **11**(1): 16.
Sadananda, P., R. Chess-Williams, et al. (2008). "Contractile properties of the pig bladder mucosa in response to neurokinin A: a role for myofibroblasts?" British Journal of Pharmacology **153**(7): 1465-1473.
Sadananda, P., F. C. L. Kao, et al. (2012). "Acid and stretch, but not capsaicin, are effective stimuli for ATP release in the porcine bladder mucosa: Are ASIC and TRPV1 receptors involved?" European Journal of Pharmacology **683**(1–3): 252-259.
Sadananda, P., F. Shang, et al. (2009). "Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch." British Journal of Pharmacology **158**(7): 1655-1662.
Sanders, K. M. and S. M. Ward (2007). "Kit mutants and gastrointestinal physiology." The Journal of Physiology **578**(1): 33-42.
Sanz-Salvador, L., A. Andrés-Borderia, et al. (2012). "Agonist- and Ca2+-dependent desensitization of TRPV1 channel targets the receptor to lysosomes for degradation." Journal of Biological Chemistry **287**(23): 19462-19471.
Säve, S. and K. Persson (2010). "Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract." Infection and Immunity **78**(8): 3609-3615.
Schaefer, M. (2005). "Homo- and heteromeric assembly of TRP channel subunits." Pflügers Archiv **451**(1): 35-42.
Schnegelsberg, B., T.-T. Sun, et al. (2010). "Overexpression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **298**(3): R534-R547.
Sculptoreanu, A., D. E. Artim, et al. (2009). "Neurokinins inhibit low threshold inactivating K+ currents in capsaicin responsive DRG neurons." Experimental Neurology **219**(2): 562-573.
Sculptoreanu, A., F. Aura Kullmann, et al. (2008). "Neurokinin 2 receptor-mediated activation of protein kinase C modulates capsaicin responses in DRG neurons from adult rats." European Journal of Neuroscience **27**(12): 3171-3181.
Sculptoreanu, A. and W. C. de Groat (2003). "Protein kinase C is involved in neurokinin receptor modulation of N- and L-type Ca2+ channels in DRG neurons of the adult rat." Journal of Neurophysiology **90**(1): 21-31.
Sculptoreanu, A. and W. C. de Groat (2007). "Neurokinins enhance excitability in capsaicin-responsive DRG neurons." Experimental Neurology **205**(1): 92-100.
Sculptoreanu, A., W. C. de Groat, et al. (2005). "Abnormal excitability in capsaicin-responsive DRG neurons from cats with feline interstitial cystitis." Experimental Neurology **193**(2): 437-443.

Sengupta, J. N. and G. F. Gebhart (1994). "Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat." Journal of Neurophysiology **72**(5): 2420-2430.

Severini, C., G. Improta, et al. (2002). "The tachykinin peptide family." Pharmacological Reviews **54**(2): 285-322.

Shabir, S., W. Cross, et al. (2013). "Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium." American Journal of Physiology - Renal Physiology **305**(3): F396-F406.

Shaker, H. S., L. M. Tu, et al. (1998). "Hyperreflexia of the urinary bladder: possible role of the efferent function of the capsaicin sensitive primary afferents." The Journal of Urology **160**(6, Part 1): 2232-2239.

Sharkey, K. A., R. G. Williams, et al. (1983). "Sensory substance P-innervation of the urinary bladder: Possible site of action of capsaicin in causing urine retention in rats." Neuroscience **10**(3): 861-868.

Shea, V. K., R. Cai, et al. (2000). "Sensory fibers of the pelvic nerve innervating the rat's urinary bladder." Journal of Neurophysiology **84**(4): 1924-1933.

Sherrington, C. S. (1892). "Notes on the arrangement of some motor fibres in the lumbo-sacral plexus." The Journal of Physiology **13**(6): 621.

Silva-Ramos, M., I. Silva, et al. (2013). "Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome." PLoS ONE **8**(5): e64696.

Silva, C., M. J. Ribeiro, et al. (2002). "The effect of intravesical resiniferatoxin in patients with idiopathic detrusor instability suggests that involuntary detrusor contractions are triggered by c-fiber input." The Journal of Urology **168**(2): 575-579.

Silva, C., M.-E. Rio, et al. (2000). "Desensitization of bladder sensory fibers by intravesical resiniferatoxin, a capsaicin analog: long–term results for the treatment of detrusor hyperreflexia." European Urology **38**(4): 444-452.

Silva, C., J. Silva, et al. (2007). "Bladder sensory desensitization decreases urinary urgency." BMC Urology **7**(1): 9.

Silva, C., J. Silva, et al. (2005). "Urodynamic effect of intravesical resiniferatoxin in patients with neurogenic detrusor overactivity of spinal origin: results of a double-blind randomized placebo-controlled trial." European Urology **48**(4): 650-655.

Skaper, S. D., P. Debetto, et al. (2010). "The P2X7 purinergic receptor: from physiology to neurological disorders." The FASEB Journal **24**(2): 337-345.

Smet, J. P, et al. (1997). Distribution and colocalization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. New York, NY, ETATS-UNIS, Nature Publishing.

Smet, P., K. Edyvane, et al. (1996). "Neuropeptides and neurotransmitter-synthesizing enzymes in intrinsic neurons of the human urinary bladder." Journal of Neurocytology **25**(1): 112-124.

Smet, P. J., K. H. Moore, et al. (1997). "Distribution and colocalization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder." Laboratory investigation; a journal of technical methods and pathology **77**(1): 37-49.

Smith, C. P., D. A. Gangitano, et al. (2008). "Botulinum toxin type A normalizes alterations in urothelial ATP and NO release induced by chronic spinal cord injury." Neurochemistry International **52**(6): 1068-1075.

Smith, C. P., V. M. Vemulakonda, et al. (2005). "Enhanced ATP release from rat bladder urothelium during chronic bladder inflammation: effect of botulinum toxin A." Neurochemistry International **47**(4): 291-297.

Stanchev, D., M. Blosa, et al. (2009). "Cross-inhibition between native and recombinant TRPV1 and P2X3 receptors." Pain **143**(1–2): 26-36.

Stein, R. J., S. Santos, et al. (2004). "Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract." The Journal of Urology **172**(3): 1175-1178.
Stewart, W., J. Van Rooyen, et al. (2003). "Prevalence and burden of overactive bladder in the United States." World Journal of Urology **20**(6): 327-336.
Strassmaier, M. and P. G. Gillespie (2002). "The hair cell's transduction channel." Current Opinion in Neurobiology **12**(4): 380-386.
Streng, T., H. E. Axelsson, et al. (2008). "Distribution and Function of the Hydrogen Sulfide–Sensitive TRPA1 Ion Channel in Rat Urinary Bladder." European Urology **53**(2): 391-400.
Su, H. C., J. Wharton, et al. (1986). "Calcitonin gene-related peptide immunoreactivity in afferent neurons supplying the urinary tract: Combined retrograde tracing and immunohistochemistry." Neuroscience **18**(3): 727-747.
Su, X., J. N. Sengupta, et al. (1997). "Effects of opioids on mechanosensitive pelvic nerve afferent fibers innervating the urinary bladder of the rat." Journal of Neurophysiology **77**(3): 1566-1580.
Sui, G.-P., C. Wu, et al. (2006). "Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts." BJU International **97**(6): 1327-1331.
Sui, G.-P., C. Wu, et al. (2008). "Modulation of bladder myofibroblast activity: implications for bladder function." American Journal of Physiology - Renal Physiology **295**(3): F688-F697.
Sui, G., C. H. Fry, et al. (2014). "Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions." American Journal of Physiology - Renal Physiology **306**(3): F286-F298.
Sui, G. P., S. Rothery, et al. (2002). "Gap junctions and connexin expression in human suburothelial interstitial cells." BJU International **90**(1): 118-129.
Sui, G. P., C. Wu, et al. (2001). "The electrophysiological properties of cultured and freshly isolated detrusor smooth muscle cells." The Journal of Urology **165**(2): 627-632.
Sui, G. P., C. Wu, et al. (2004). "Electrical characteristics of suburothelial cells isolated from the human bladder." The Journal of Urology **171**(2, Part 1): 938-943.
Suman-Chauhan, N., S. Guard, et al. (1990). "Pharmacological characterization of tachykinin-stimulated inositol phospholipid hydrolysis in peripheral tissues." British Journal of Pharmacology **101**(4): 1001-1005.
Sun, B., Q. Li, et al. (2010). "Ion channel and receptor mechanisms of bladder afferent nerve sensitivity." Autonomic Neuroscience **153**(1–2): 26-32.
Sun, Y. and T. C. Chai (2002). "Effects of dimethyl sulphoxide and heparin on stretch-activated ATP release by bladder urothelial cells from patients with interstitial cystitis." BJU International **90**(4): 381-385.
Sun, Y. and T. C. Chai (2005). "Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis." American Journal of Physiology - Cell Physiology **290**(1): C27-C34.
Sun, Y. A. N. and T. C. Chai (2004). "Up-regulation of P2X3 receptor during stretch of bladder urothelial cells from patients with interstitial cystitis." The Journal of Urology **171**(1): 448-452.
Sun, Y. A. N., S. Keay, et al. (2001). "Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis." The Journal of Urology **166**(5): 1951-1956.
Tanaka, I., K. Nagase, et al. (2011). "Modulation of stretch evoked adenosine triphosphate release from bladder epithelium by prostaglandin E2." The Journal of Urology **185**(1): 341-346.
Tempest, H. V., A. K. Dixon, et al. (2004). "P2X2 and P2X3 receptor expression in human bladder urothelium and changes in interstitial cystitis." BJU International **93**(9): 1344-1348.
Templeman, L., D. J. Sellers, et al. (2003). "Investigation of neurokinin-2 and -3 receptors in the human and pig bladder." BJU International **92**(7): 787-792.

Thor, K. B., C. Morgan, et al. (1989). "Organization of afferent and efferent pathways in the pudendal nerve of the female cat." The Journal of Comparative Neurology **288**(2): 263-279.

Timóteo, M. A., I. Carneiro, et al. (2014). "ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors." Biochemical Pharmacology **87**(2): 371-379.

Tominaga, M., M. J. Caterina, et al. (1998). "The cloned capsaicin receptor integrates multiple pain-producing stimuli." Neuron **21**(3): 531-543.

Tominaga, M. and T. Tominaga (2005). "Structure and function of TRPV1." Pflügers Archiv **451**(1): 143-150.

Tominaga, M., M. Wada, et al. (2001). "Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia." Proceedings of the National Academy of Sciences **98**(12): 6951-6956.

Tompkins, J., B. Girard, et al. (2010). "VIP and PACAP effects on mouse major pelvic ganglia neurons." Journal of Molecular Neuroscience **42**(3): 390-396.

Torrens, Y., J.-C. Beaujouan, et al. (1995). "Involvement of septide-sensitive tachykinin receptors in inositol phospholipid hydrolysis in the rat urinary bladder." Peptides **16**(4): 587-594.

Torrisi, G., E. G. Sampugnaro, et al. (2007). "Postpartum urinary stress incontinence: analysis of the associated risk factors and neurophysiological tests." Minerva ginecologica **59**(5): 491-498.

Tramontana, M., R. M. Catalioto, et al. (2000). "Role of prostanoids in the contraction induced by a tachykinin NK2 receptor agonist in the hamster urinary bladder." Naunyn-Schmiedeberg's Archives of Pharmacology **361**(4): 452-459.

Truschel, S. T., E. Wang, et al. (2002). "Stretch-regulated exocytosis/endocytosis in bladder umbrella cells." Molecular Biology of the Cell **13**(3): 830-846.

Tucci, P., P. Bolle, et al. (2001). "Effects of natural tachykinins on ovine lower urinary tract smooth muscle." Journal of Autonomic Pharmacology **21**(2): 79-84.

Tyagi, P., V. Tyagi, et al. (2013). "Association of inflammaging (inflammation + aging) with higher prevalence of OAB in elderly population." International Urology and Nephrology: 1-7.

Ueno, S., M. Tsuda, et al. (1999). "Cell type-specific ATP-activated responses in rat dorsal root ganglion neurons." British Journal of Pharmacology **126**(2): 429-436.

Veenboer, P. W. and J. L. H. R. Bosch (2013). "Long-term adherence to antimuscarinic therapy in everyday practice: a systematic review." The Journal of Urology(0).

Vera, P. L., K. A. Iczkowski, et al. (2008). "Cyclophosphamide-induced cystitis increases bladder CXCR4 expression and CXCR4-macrophage migration inhibitory factor association." PLoS ONE **3**(12): e3898.

Vera, P. L. and I. Nadelhaft (1990). "Conduction velocity distribution of afferent fibers innervating the rat urinary bladder." Brain Research **520**(1–2): 83-89.

Virginio, C., A. MacKenzie, et al. (1999). "Pore dilation of neuronal P2X receptor channels." Nat Neurosci **2**(4): 315-321.

Viviani, B., F. Gardoni, et al. (2007). Cytokines and neuronal ion channels in health and disease. International Review of Neurobiology. M. T. C. Giacinto Bagetta and A. L. Stuart, Academic Press. **Volume 82:** 247-263.

Vizzard, M. A. (2000). "Alterations in spinal cord Fos protein expression induced by bladder stimulation following cystitis." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **278**(4): R1027-R1039.

Vlaskovska, M., L. Kasakov, et al. (2001). "P2X3 knock-out mice reveal a major sensory role for urothelially released ATP." The Journal of Neuroscience **21**(15): 5670-5677.

von Kügelgen, I. (2006). "Pharmacological profiles of cloned mammalian P2Y-receptor subtypes." Pharmacology & Therapeutics **110**(3): 415-432.

Wagg, A., G. Compion, et al. (2012). "Persistence with prescribed antimuscarinic therapy for overactive bladder: a UK experience." BJU International **110**(11): 1767-1774.

Wang, E., S. Truschel, et al. (2003). "Analysis of hydrostatic pressure-induced changes in umbrella cell surface area." Methods **30**(3): 207-217.
Wang, E. C. Y., J.-M. Lee, et al. (2005). "ATP and purinergic receptor–dependent membrane traffic in bladder umbrella cells." The Journal of Clinical Investigation **115**(9): 2412-2422.
Wang, N., A. Orr-Urtreger, et al. (2002). "The role of neuronal nicotinic acetylcholine receptor subunits in autonomic ganglia: lessons from knockout mice." Progress in Neurobiology **68**(5): 341-360.
Wang, Z.-Y., P. Wang, et al. (2008). "Lack of TRPV1 inhibits cystitis-induced increased mechanical sensitivity in mice." PAIN **139**(1): 158-167.
Wein, A. J. and E. S. Rovner (2002). "Definition and epidemiology of overactive bladder." Urology **60**(5, Supplement 1): 7-12.
Wibberley, A., Z. Chen, et al. (2003). "Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle." British Journal of Pharmacology **138**(5): 757-766.
Winter, J. (2005). TRPV1 distribution and regulation. Turning up the Heat on Pain: TRPV1 Receptors in Pain and Inflammation. A. Malmberg and K. Bley, Birkhäuser Basel**:** 39-51.
Wiseman, O. J., C. J. Fowler, et al. (2003). "The role of the human bladder lamina propria myofibroblast." BJU International **91**(1): 89-93.
Wu, C., G. P. Sui, et al. (2004). "Purinergic regulation of guinea pig suburothelial myofibroblasts." The Journal of Physiology **559**(1): 231-243.
Xu, L. and G. F. Gebhart (2008). "Characterization of mouse lumbar splanchnic and pelvic nerve urinary bladder mechanosensory afferents." Journal of Neurophysiology **99**(1): 244-253.
Xu, X., E. A. Gordon, et al. (2009). "Functional TRPV4 channels and an absence of capsaicin-evoked currents in freshly-isolated, guinea-pig urothelial cells." Channels **3**(3): 156-160.
Yamada, T., S. Ugawa, et al. (2009). "Differential localizations of the transient receptor potential channels TRPV4 and TRPV1 in the mouse urinary bladder." Journal of Histochemistry & Cytochemistry **57**(3): 277-287.
Yang, Y.-L., K.-H. Yao, et al. (2003). "Similarities of SP-, NKA- and NKB-induced currents in rat dorsal root ganglion neurons." Brain Research **991**(1–2): 18-25.
Yiangou, Y., P. Facer, et al. (2001). "Capsaicin receptor VR1 and ATP-gated ion channel P2X3 in human urinary bladder." BJU International **87**(9): 774-779.
Yokoyama, O., I. Tanaka, et al. (2011). "Antimuscarinics Suppress Adenosine Triphosphate and Prostaglandin E2 Release From Urothelium With Potential Improvement in Detrusor Overactivity in Rats With Cerebral Infarction." The Journal of Urology **185**(6): 2392-2397.
Yokoyama, O., M. Yoshiyama, et al. (2000). "Role of the forebrain in bladder overactivity following cerebral infarction in the rat." Experimental Neurology **163**(2): 469-476.
Yoshida, M., Y. Homma, et al. (2001). "Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles." Experimental Gerontology **36**(1): 99-109.
Yoshida, M., A. Inadome, et al. (2006). "Non-neuronal cholinergic system in human bladder urothelium." Urology **67**(2): 425-430.
Yoshida, M., K. Miyamae, et al. (2004). "Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging." Urology **63**(3, Supplement 1): 17-23.
Yoshimura, N., Y. Kaiho, et al. (2008). "Therapeutic receptor targets for lower urinary tract dysfunction." Naunyn-Schmiedeberg's Archives of Pharmacology **377**(4-6): 437-448.
Yoshimura, N., S. Seki, et al. (2003). "Histological and electrical properties of rat dorsal root ganglion neurons innervating the lower urinary tract." The Journal of Neuroscience **23**(10): 4355-4361.
Yu, W. and W. G. Hill (2011). "Defining protein expression in the urothelium: a problem of more than transitional interest." American Journal of Physiology - Renal Physiology **301**(5): F932-F942.
Yu, W., S. C. Robson, et al. (2011). "Expression and distribution of ectonucleotidases in mouse urinary bladder." PLoS ONE **6**(4): e18704.

Yu, W., L. C. Zacharia, et al. (2006). "Adenosine receptor expression and function in bladder uroepithelium." American Journal of Physiology - Cell Physiology **291**(2): C254-C265.

Yu, Y. and W. C. de Groat (2008). "Sensitization of pelvic afferent nerves in the in vitro rat urinary bladder-pelvic nerve preparation by purinergic agonists and cyclophosphamide pretreatment." American Journal of Physiology - Renal Physiology **294**(5): F1146-F1156.

Zagorodnyuk, V. P., S. J. H. Brookes, et al. (2010). "Structure–function relationship of sensory endings in the gut and bladder." Autonomic Neuroscience **153**(1–2): 3-11.

Zagorodnyuk, V. P., S. J. H. Brookes, et al. (2009). "Mechanotransduction and chemosensitivity of two major classes of bladder afferents with endings in the vicinity to the urothelium." The Journal of Physiology **587**(14): 3523-3538.

Zagorodnyuk, V. P., M. Costa, et al. (2006). "Major classes of sensory neurons to the urinary bladder." Autonomic Neuroscience **126–127**(0): 390-397.

Zagorodnyuk, V. P., I. L. Gibbins, et al. (2007). "Properties of the major classes of mechanoreceptors in the guinea pig bladder." The Journal of Physiology **585**(1): 147-163.

Zagorodnyuk, V. P., P. Lynn, et al. (2005). "Mechanisms of mechanotransduction by specialized low-threshold mechanoreceptors in the guinea pig rectum." American Journal of Physiology - Gastrointestinal and Liver Physiology **289**(3): G397-G406.

Zarghooni, S., J. Wunsch, et al. (2007). "Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium." Life Sciences **80**(24–25): 2308-2313.

Zhang, H., C.-L. Cang, et al. (2007). "Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCε: a novel pathway for heat hyperalgesia." The Journal of Neuroscience **27**(44): 12067-12077.

Zhang, X., J. Huang, et al. (2005). "NGF rapidly increases membrane expression of TRPV1 heat‐gated ion channels." The EMBO Journal **24**(24): 4211-4223.

Zhong, Y., P. M. Dunn, et al. (2001). "Changes in P2X receptor responses of sensory neurons from P2X3-deficient mice." European Journal of Neuroscience **14**(11): 1784-1792.

Zygmunt, P. M., J. Petersson, et al. (1999). "Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide." Nature **400**(6743): 452-457.